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CONTENTS

Cover: Yearling coho salmon, *Oncorhynchus kisutch*, are vaccinated against vibriosis using some of the first production techniques developed by NMFS biologists for the commercial salmon farming industry.

Health, Disease, and Disease Prevention in Cultured Aquatic Animals

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Preface

Aquaculture in the northern hemisphere has been increasing at a rapid pace. This is evident from the increasing tonnage of fish produced, the culture of additional species, and the development of new techniques to culture aquatic animals. Worldwide, aquacultural production is now approaching 10 percent of the total fishery products harvested by all methods. Concomitantly in the United States, people in the aquaculture industry began to recognize that they were sharing the same problems as those people in intensive agriculture, and disease is a particularly important problem of common interest.

In 1975, Norden Laboratories of Lincoln, Neb., sponsored a unique and wide-ranging symposium. Its purpose was to share scientific and technical knowledge in controlling diseases of cultured aquatic animals. However, rather than bringing together a conclave of people solely interested in fisheries, Norden included veterinarians, chemists, and technologists involved in veterinary medicine, and government scientists and administrators concerned with the control of biologics, pharmaceuticals, and chemotherapeutic agents in agriculture. The papers which follow were among those presented at the symposium, and were selected for *Marine Fisheries Review* strictly on the basis of their application to aquaculture.

The editors are grateful to Pat Pike of Norden Laboratories for the time and effort she spent after the symposium, in the initial phases of working with the authors of these papers to get them ready for publication.

Thanks are also due to the authors for their loyalty and patience while we gathered the fruits of their labor for publication in this journal. The work carried out by Carol Oswald and Steve Jensen of the graphics group of the NMFS Northwest and Alaska Fisheries Center, in clarifying some of the graphs and figures is gratefully acknowledged. The final reading of the papers by Lee W. Harrell was a big help. Lastly, recognition is given to Norden Laboratories for sponsoring and hosting the symposium.

Anthony J. Novotny
M. Michael Sigel

Immunity and Practical Vaccine Development

ALBERT L. BROWN

ABSTRACT — The practical aspects of veterinary vaccine development and how vaccines may be designed better to protect animals against disease are reviewed. It is emphasized that vaccine development must be directed toward producing the type of immune response required to protect against a particular disease. In attempting to protect fish through vaccination, there are new opportunities and problems to be considered: The temperature dependent immune response, isolation problems, importance of group immunization VS individual protection, and the concept of breeding fish capable of producing a superior immune response.

The work done by research and development groups in commercial organizations that prepare vaccines has much in common with the work done by laboratories of government and academic institutions in that some of their activities might be considered basic science. The scientific personnel of these commercial organizations are working at about the same level of competence as their colleagues in veterinary schools and the veterinary science departments. While vaccine development may not be a science, it is certainly sophisticated technology. The day is long past when it was possible to throw together the ingredients for a vaccine, test it in a few animals, demonstrate some level of protection, and call the result a vaccine. The public's expectations of effectiveness have increased; these expectations are reinforced and strengthened by appropriate government regulations. Although the public's expectations may be excessive, there is absolutely no question that

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vaccines today are much better than they were in 1950.

TYPES OF VACCINES

Basically, we might say that there are five kinds of veterinary vaccines: 1) Inactivated bacterial vaccines (referred to as bacterins); 2) modified live bacterial vaccines; 3) toxoids; 4) inactivated viral vaccines; and 5) modified live viral vaccines.

There are also vaccines against species of ricksettsia, coccidia, hookworms, and other agents. Some antiserum is still used. In the past, combinations of virulent virus and antiserum were used for some diseases, but this practice has been discontinued.

TYPES OF IMMUNITY

In birds and mammals, the immune system may be conveniently divided into two components: Humoral immunity and cell mediated immunity. These two components serve to supplement each other and appear to naturally regulate each other. Humoral immunity involves a synthesis and release of free antibody into the blood and other body fluids. Antibody acts by direct combination with the antigen. Humoral immunity is most effective in neutralizing toxins and by coating bacteria to enhance their phagocytosis. It also plays a part in neutralizing certain viruses. Cell mediated immunity involves a production of specifically sensitized cells and is expressed by such reactions as rejection of skin transplants, delayed type hypersensitivity, and the destruction of cells infected with viruses or bacteria. Cell mediated immunity is considered to be the body's primary response against cells such as cancer cells.

INACTIVATED VERSUS LIVE VACCINES

Before developing a vaccine, a clear-cut idea of the type of protection that is needed is helpful. If the disease is caused by a toxin, inoculation of toxoid or adjuvanted toxoid to stimulate humoral immunity is the route to follow. In this case fractionation and purification of bacterial antigens are inappropriate. If the disease is caused by a virus, inoculation of modified live virus which serves to stimulate both humoral and cell mediated immunity is probably the route to follow. Vaccines which have generally been most satisfactory fall into the two mentioned categories. There are exceptions but generally speaking, long-lasting and strong immunity has not been the strong point of inactivated bacterial vaccines

and inactivated viral vaccines. Veterinarians and physicians indicate their preference for modified live virus vaccines when they have a choice. Properly researched and prepared, I believe in modified live virus vaccines—there is less chance of the modified virus reverting to virulence by mutation than there is for inactivated viral vaccines to contain virulent virus because of failure to be fully inactivated. There have been some widely publicized examples of so-called inactivated viral vaccines which have not been inactivated. As capable a scientist as Jonas Salk failed to recognize all of the parameters involved in the inactivation of poliomyelitis virus. After the tragic results from use of improperly inactivated commercial vaccine, the entire procedure was reexamined by Sven Gard. He found that too rapid inactivation with formaldehyde served to denature the viral capsid protein. By not allowing the proper concentration of formaldehyde to enter into the interior of the virus, the viral nucleic acid was not inactivated. At this microlocation, the predetermined inactivation curve did not apply.

DEVELOPING AN INACTIVATED BACTERIAL VACCINE

We have much to consider when we start to develop a vaccine. I will present some of the points to be considered in developing an inactivated bacterial vaccine.

What is the etiological agent?

Which bacteria are important in the disease under study and how many strains are there? Is this a disease situation where four or five different strains are required for coverage or only one? Once identified it becomes necessary to select antigenic strains.

A production method retaining antigenicity must be developed.

A growth cycle has to be developed. This involves determining the time, the temperature, the culture medium, the oxygen content, whether a fermentor is feasible, and how the culture will be stored.

A method of inactivation has to be developed, but not overinactivation to destroy the antigen. It must be determined whether inactivation is going to be a chemical procedure or a physical procedure. Often it will be a combination of both.

It must be determined if there will be a fractionation or concentration procedure and how to accomplish it.

It must be determined if an adjuvant is useful.

Control procedures to measure potency have to be developed.

At this point it is necessary to have the usual control procedures for safety, purity, and sterility involving in vitro and host animal and laboratory animal inoculation.

The best route of inoculation has to be determined. Not all routes of inoculation are equally effective for all vaccines.

Dosage has to be determined, not only the number of milliliters to be inoculated, but how much antigen per milliliter.

The number of doses required to stimulate what degree of immunity must be determined. Can effective immunization be achieved with one dose or will it take two doses, or three doses, and how are they to be spaced?

It is necessary to determine how rapidly it will be possible to develop immunity, the duration of immunity, the revaccination schedule, when that is going to be, a year following, two years following, and how often. More vaccination is not necessarily better.

Each inoculation is a medical procedure and not entirely free from risk. There are side reactions following vaccination. The dangers of hypersensitivity must be considered.

The practical considerations of production are necessary, such as: Single dose or multiple dose packaging; how the product should be protected against contamination from misuse; uniformity in appearance and packaging; ease of administration; respectable shelf life with retained potency; quality of bottles, stoppers, and seals; and documentation and support by host animal efficacy studies, and field trials, to uncover the unexpected.

DEVELOPING MODIFIED LIVE BACTERIAL VACCINES

Most of the same things have to be considered for developing modified live bacterial vaccines as for inactivated bacterial vaccines and, in addition, there are some other points to consider. Of foremost importance is the degree of attenuation required to achieve a balance between effectiveness and safety. We must be able to modify the bacteria so it will have no ability to produce disease nor tend toward reversion. Modified live bacterial vaccines are generally standardized by count. What that count will have to be has to be determined. The vaccine will probably have to be lyophilized to achieve useful shelf life, that is, vacuum dried from the frozen state. A stabilizer will have to be developed.

DEVELOPING VIRAL VACCINES

For viral vaccines, we have still further complications. The substrate on which the virus is grown is equally as important as the virus itself. Viruses only grow in living cells and if molecular biology has anything to teach us, it is that a virus-infected cell is a new biological entity different from the cell you started with and different from the virus. Originally, viral vaccines were produced from tissues or blood of infected animals. Normal, healthy animals were inoculated with virus. When they became sick, the tissues were removed, ground up, and inactivated. This was considered to be a virus vaccine. This was followed by the use of embryonated eggs—substituting them for animals. Following the discovery that low levels of antibiotics would control contamination in tissue culture, tissue culture gradually became the method of choice.

Originally, there was a great deal of difficulty in producing sterile and pure viral vaccines. This was because of latent infections in the cells as well as contamination being introduced in the process. While sterility and purity are not necessarily a problem with inactivated vaccines, it is a problem with

Standard Seed Virus + Primary Cells from variety of sources

STANDARD CONDITIONS
Culture Medium
Temperature
Time

Virus for Vaccine Production

VARIES FROM SERIAL TO SERIAL

Standard Seed Virus + Stable Cell Line

STANDARD CONDITIONS
Culture Medium
Temperature
Time

Virus for Vaccine Production
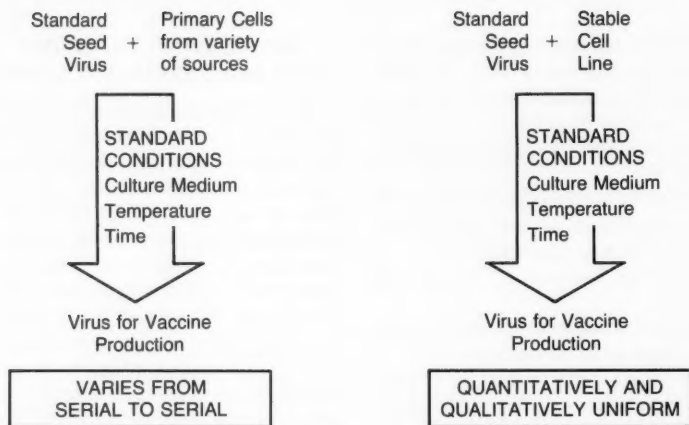
QUANTITATIVELY AND QUALITATIVELY UNIFORM

Figure 1.—Comparison of two procedures for obtaining virus for vaccine production.

modified live virus vaccines. Along with the attenuated virus, almost any other virus might be found in the tissues of the host being used as a source of cells. Examples of this may be found with all types of viral propagation. It is instructive that originally, the inactivated polio vaccine was contaminated with SV-40 virus. The SV-40 virus is found widely in stocks of rhesus monkeys and is latent in their kidney cells. How do you look for something that you do not even know about? Nobody looked for SV-40 virus in the earliest days of polio vaccine production. Furthermore, it was more difficult to inactivate than was poliomyelitis virus and consequently, some live SV-40 was inadvertently introduced into many lots of vaccine used to inoculate people. Sometime later it was discovered that SV-40 virus was capable of transforming human cells in tissue culture. So far, there is no evidence SV-40 virus has ever produced any transformations in people. Contamination of viral vaccine is not just peculiar to tissue culture. It is something that has been with us a long time. For example, the last foot and mouth disease outbreak in the United States was traced to importation of smallpox vaccine, which was used to inoculate a farmer, the farmer spread it to his calves. The calves used to produce the smallpox vaccine must have had foot and mouth disease.

STABLE CELL LINE DEVELOPMENT

It has become customary in the last few years to use pretested cells for tissue culture vaccine production. The checking of these cells is just about as complex as the development of a bacterial vaccine. I feel the best procedure is to use a stable cell line. By this procedure, it is possible to work with essentially the same cells over a long period of time. These cells are preserved by freezing away a master seed stock. Cells may be recovered from this source and used for many years to grow up cells for vaccine production. Figure 1 illustrates this point. From the beginning of virus vaccine production it has been customary to use a standard seed virus. There is no problem in maintaining such constants as time, temperature, and culture medium. The problem has been the inability to guarantee a good source of primary cells, whether we worked from animals, embryonated eggs, or primary tissue cultures. When unchecked cells are used as the source of virus, vaccine is going to vary from serial to serial. This whole situation can be greatly improved by using an established cell line. By adding an established cell line into the equation it is possible to produce virus for vaccine production which is quantitatively and qualitatively uniform from serial to serial.

The cells of an established cell line are all genetically related. Within the limits of biological variation, they all have the same biochemical characteristics, the same ability to absorb virus, the same response to the virus, and will produce virus quantitatively and qualitatively equal each time the procedure is followed in detail.

These details seem to be involved and complicated, but someplace in any vaccine development program they must be considered.

DEVELOPING FISH VACCINES

Temperature Dependent Immune Response

The immune response in fish is temperature dependent. This is a new complication. Generally speaking, mammals and birds all have a uniform body temperature. The safety of attenuated products has to be considered in terms of this temperature dependence.

Isolation Problems

Another problem I foresee in working with fish vaccines is the lack of isolation with animals living in water. With land animals we are not too concerned with spreading virus. Fresh air provides a big dilution factor. At Pirbright, England, where foot and mouth disease is studied, it is documented that virus escaped and was airborn to another place miles away but this is the exception. Fish are more intimately involved with their environment than land animals. Water can provide a medium for parasites and diseases that can spread to other species through very complex biological cycles.

Group (Not Individual) Vaccination Needed

In vaccinating dogs, it is necessary to protect every dog, or almost every dog, that is brought into the veterinarian's office. We have to be able to do this without regard to breed, age, sex, the health of the dog, and in spite of intercurrent infections, various medications and general husbandry. Some dogs, of

course, are unable to respond. Each dog is treated as an individual. Everyone expects 100 percent success. There are techniques and information which would permit dog breeders to breed dogs for resistance to canine distemper or away from the tendency to contract hepatitis, but these are problems on which dog breeders prefer not to work. Dog breeders are interested in conformation, temperament, hip dysplasia, trainability, and hunting instinct. They have a whole host of things to worry about other than immunization. The manufacturers of veterinary biologics are taking care of that for them.

With cattle we tend to consider them more in groups. There is still a great diversity in their backgrounds and it is expected vaccination will protect nearly 100 percent of the cattle. Although we suspect not all breeds of cattle immunize equally well, nobody is worried about breeding cattle for ability to immunize. In fact, some of our practices may actually work against selection of cattle able to produce a good immune response. However, people generally are concerned about cattle as individuals and expect to have a high degree of success. Nobody seems to worry about chickens as individuals. I see fish as being some place between poultry and field crops as far as being recognized as individuals. What we are concerned about in fish vaccination is the final result. Accordingly, I do not believe it should be necessary to protect virtually every individual providing the end result will produce a profit for the fish farmer. This is successful farming.

New Concept for Successful Fish Immunization

I can foresee with fish culture that if it is necessary to protect just any fish regardless of how they are raised or handled it is going to be a difficult situation. There are things in fish culture that can
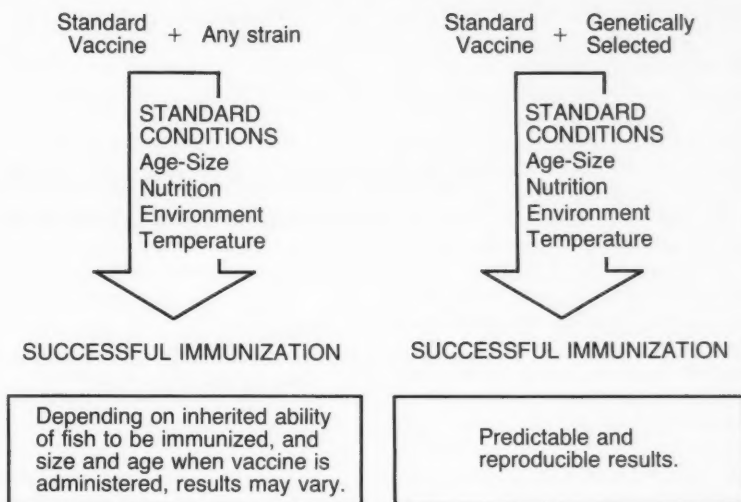
Figure 2.—Comparison of two procedures for immunization of fish.

be controlled, such as: The date of fertilization; physiological age; conditions of nutrition and environment; and genetics. If vaccine is to be administered in the water, the water can be adjusted for the chemical composition, temperature, and pH. It is possible to obtain many fish from one mating and I think the genetics of fish could be something that should be studied. It would be interesting to combine genetics with immunization and produce strains of fish which will produce a good immune response. We could either develop vaccines for the fish or the fish for the vaccine. Figure 2 shows how this fits into a scheme for obtaining more uniform response. By substituting stan-

dard vaccines for standard seed virus and substituting genetically selected fish for the stable cell line, it might be possible to move the concept of uniformly successful immunization one step further.

CONCLUSION

I would like to point out that researchers in commercial organizations preparing vaccines have a certain expertise and the aquatic scientists at this meeting have another. Perhaps, by pooling our scientific information and technology we might be able to progress more rapidly in the development of vaccines for fish.

Cellular Immunity in Fish as Measured by Lymphocyte Stimulation

M. MICHAEL SIGEL, JOHN C. LEE, E. CHURCHILL McKINNEY,
and DIANA M. LOPEZ

ABSTRACT—Fish are capable of responding to a variety of antigens. In many instances the primary response has attributes similar to those of mammals or birds, but in other ways the immunologic responses of fish stand apart, e.g., fish produce only one major class of immunoglobulin (IgM). Fish appear to handle all antigens as if they were thymus-independent. Some species have a faulty or nonexistent immunologic memory. Although fish lymphocytes perform certain functions characteristic of T or B cells of mammals, there is no clear-cut evidence that these are performed by specialized lymphocytes as opposed to lymphocytes with multiple functions. Several factors appear to be capable of regulating immune response. These include the IgM natural antibodies (some of which have nonspecific immunologic reactivity), immune complexes, and suppressor cells. All of these may combine to suppress certain responses. It therefore behooves the profession to undertake more extensive and intensive studies in fish immunology if the profession is to develop a better understanding of optimal modes and conditions for achieving protective immunity in fish.

Improvements and refinements in methodologies of tissue culture, immunology, virology, and bacteriology have made possible the attainment of new knowledge regarding the diseases of fish. A deeper appreciation of the factors contributing to the health of these poikilothermic animals has come from immunological studies. More recently, various parameters of cell-mediated immunity have come under scrutiny. It has been known for some time, and confirmed in our laboratory, that bony fishes are capable of rejecting allografts (Hildemann, 1957; Hildemann and Haas, 1960; Hildemann and Cooper, 1963). The rejection process in these animals is fairly acute. A more chronic process occurs in the shark (Hildemann, 1970). Allograft rejection in higher forms is mediated by lymphocytes activated by histocompatibility antigens which can be demonstrated in vitro by the blastogenic reaction. This is a complex reaction which is manifested in a variety of ways — increased permeability of lymphocyte membranes and elevation of the rate of synthesis of protein, RNA, and DNA. The biochemical changes are accompanied by an enlargement of cells whereby the lymphocytes revert to lymphoblasts, which can then reproduce by mitosis.

All these changes can be measured by biochemical or morphological methods, and the most commonly employed method measures DNA synthesis (incorporation of radioactive thymidine into DNA). Blastogenic transformation reactions occur as a result of activation of lymphocytes by histocompatibility antigens (antigens involved in graft rejection), as just mentioned, or as a result of lymphocyte stimulation by specific nontissue antigens, i.e., viral, bacterial, etc., or nonspecific mitogenic lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) or certain carbohydrates or lipopolysaccharides (LPS) in bacterial endotoxins.

Antigens can bind to specific receptors present on a few lymphocytes in the nonimmunized host and to the clones of lymphocytes following immunization with this antigen. This is believed to be the mode of expansion of a clone of lymphocytes specific for the antigen, assuming that the antigen is bound to the recognition receptor on the lymphocyte membrane, and this event generates a signal for a blastogenic transformation culminating in lymphocyte proliferation. The progeny lymphocytes recognize and respond (with blastogenic transformation) to the same antigen that launched the initial selective stimulation of the progenitor lymphocyte. This is a simplified version of an immunologic pyramidal reaction which does not take into account a variety of regulatory factors: proliferative asymmetry, suppressor cells, temporary anergy, etc. Moreover, knowl-

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edge about blastogenic transformation is derived mainly from in vitro studies, and relatively little is known about its occurrence and character in vivo. While blastogenic transformation induced by an antigen expresses a specific response, blastogenic transformation evoked by lectins or LPS is a more general response of large segments of lymphocytes regardless of specificity, commitment, or immune status. The only rule that seems to apply here is that T lymphocytes respond to PHA and Con A, and B lymphocytes react to LPS.

Following this introduction to blastogenic reactions let me return briefly to my initial comments about allograft rejection. As has been stated, fish reject allografts, and some fish reject them as rapidly as do mammals, but the analogy does not appear to extend to the blastogenic reaction. When the lymphocytes of two unidentical mice, A and B, are placed in culture they will react blastogenically — A VS B and B VS A. In contrast, when the lymphocytes of two snappers, A and B, are placed in culture they do not react in this manner. The reason for this discrepancy is not known. However, fish lymphocytes do respond with blastogenic reaction to certain antigens, and I will discuss this after a brief statement about the immunoglobulins and an overview of the immunologic response in fish.

IMMUNOGLOBULINS

We and others have shown that fishes, except for the lungfishes, possess only one kind of immunoglobulin, IgM (Clem and Sigel, 1963; Fish et al., 1966; Clem et al., 1967; Marchalonis and Edelman, 1968; Pollara et al., 1968). However, this single class of immunoglobulin exists in several physical states. It may exist as a 7S monomer, as a 14S tetramer, or as a 19S pentamer. To our knowledge, no one has conclusively shown the presence of other immunoglobulins, such as IgA, IgG, or IgD in fishes (except perhaps lungfishes). This was the main reason that William Clem and I were led to think that fishes were immunologically simple. But, as it developed in the course of our studies,

even this single major immunoglobulin class has presented some rather challenging and interesting problems. I shall not go further into descriptions of immunoglobulins as this has been done in numerous reviews (Sigel et al., 1970; Clem, 1971; Hildemann and Clem, 1971; DuPasquier, 1973; Sigel, 1974). In this presentation I shall be more concerned with immunologic memory, regulation of the immune response, and cellular immunity.

IMMUNOLOGIC RESPONSE

By and large, the primary immune response is relatively efficient. One can evoke significant primary responses to many antigens provided the temperature is conducive or permissive to immunization. It has been recognized for a long time that the efficiency of immunization depends on the temperature of the water. Some very elegant studies on this problem have been conducted by Avtalion et al., (1973). Assuming that the temperature of the water is optimal or close to optimal, one can expect positive responses to primary immunization. As regards immunologic memory, the problem becomes more complicated. With some antigens, and in some species, there is a strong secondary response (Ridgeway et al., 1966). On the other hand, there are fishes which fail to respond to secondary stimulation. For example, in the sharks studied in our laboratory it was very difficult to elicit a secondary immune response at 1 month, 3 months, or 9 months after a successful primary immunization (Sigel and Clem, 1966). Only when the primary response was weak was it possible to elicit a heightened secondary response. If the primary response was strong, the secondary response would usually lack vigor, intensity, and amplitude.

Why this deficiency? Inability to make IgG has been suggested as an explanation, but teleosts are quite competent in mounting anamnestic responses even though they, too, fail to form IgG. The absence of a differentiated thymus gland is another possible explanation. Fishes do possess thymic glands, but these are rather primitive organs, resembling lymph nodes, and

are virtually devoid of epithelial structures. In some ways the shark's immunologic response resembles the mammalian response to a thymus-independent antigen. One of modern immunology's dogmas holds that antibody production is a funtion of B cells — a class of lymphocytes characterized by their ability to synthesize immunoglobulins. Contained in this dogma is the precept that this B cell function requires the cooperation and help of thymus-derived lymphocytes, the T cells. But this is not an absolute requirement and there exist antigens which apparently succeed in stimulating B cells toward antibody production without the help of T cells. These are designated as thymus-independent antigens, in contrast to those which require helper T cells. One such antigen is pneumococcus polysaccharide. This thymus-independent antigen does not evoke a true secondary response in mice (Baker et al., 1970). It is tempting therefore to speculate that, in the absence of a mature (differentiated) thymus gland, any antigen can direct the immune mechanism in a manner analogous to the direction provided by thymus-independent antigen. What I am proposing is that, on the one hand the shark lacks helper T cells, and on the other that its B cells seem to respond to antigens which in higher animals require the helper function of T cells. This would imply that lymphocytes of fishes (at least some fishes) may not fit precisely into the categories created for mouse or human T and B cells. Moreover, it is possible that in phylogenetically lower classes some lymphocytes may perform both T and B functions or at least some of these functions.

REGULATION OF IMMUNE RESPONSE

One of the regulatory mechanisms in fish immunity probably resides in natural antibodies with reactivity directed to a variety of antigens. The shark is remarkable in this respect. The natural antibody, although an immunoglobulin and constructed like the typical 19S IgM antibody molecule, differs from the antibody raised by im-

munization in that it possesses an unusually broad specificity (Sigel et al., 1970). For example, antibodies prepared in rabbits by immunization against chicken red blood cells (CRBC) react with CRBC but not sheep RBC (SRBC) owing to narrow specificity and the lack of detectable cross-reactive antigens. The natural antibodies of sharks, on the other hand, react with a large array of RBC's — human, pigeon, chicken, rabbit, sheep, and even such exotic animals as the tapir. These antibodies also kill bacteria and neutralize viruses of humans (influenza) and chickens (Rous sarcoma). They also bind small haptens (Sigel et al., 1970). The origin of this polyreactivity is not known. What is even more remarkable is that the multiplicity of reactivity is resident in single antibody molecules. That is to say that individual molecules of 19S IgM react with multiple antigens. One way to show this is by mixed hemagglutination reaction as illustrated in Figure 1. Serum from an unimmunized shark is added to CRBC and the natural antibody is allowed to bind to the cells. After a short incubation, the cells are washed to remove free antibody and are mixed with SRBC which have not been exposed to antibody. The occurrence of agglutinated clumps or rosettes wherein CRBC attract on to themselves SRBC indicates that the antibody on the CRBC also forms a linkage with SRBC. This signifies that the shark natural antibody can bind to at least two distinctive antigens. Such dual specificity is usually not observed in antibodies raised by immunization. Other types of determinations based on antibody isolation by means of immunoadsorbents have led to similar conclusions: the natural antibody of the shark possesses polyspecificity. Such antibodies are likely to modify or regulate the immune response. They may deplete antigen to subimmunogenic levels. Alternately, natural antibody could conceivably change the physical state of the antigen, i.e., degree of dispersion or type of configuration rendering it more or less immunogenic. However, the most profound regulatory action is probably exerted by antibody-antigen complexes which will be discussed later.

EVIDENCE THAT ANTIBODIES CAN REGULATE LYMPHOCYTE RESPONSES

Studies aimed at elucidating the role of antibody in regulating lymphocyte functions were conducted in a large series of experiments based on blastogenic transformation reactions of lymphocytes of sharks, snappers, and rabbits. In these studies, we principally used specific antibodies raised by immunization. The response of lymphocytes from immune sharks to specific antigens is illustrated in Figure 2. Shark No. 5520 was immunized with bovine gamma globulin (BGG) and shark No. 7201 with poliovirus. Peripheral blood lymphocytes were cultured at 24°C in the presence of different concentrations of BGG or poliovirus. The lymphocytes of shark 5520 reacted specifically to BGG but not to poliovirus whereas the lymphocytes of shark 7201 reacted to poliovirus but not to BGG. A mixture of both antigens caused stimulation of lymphocytes in both sharks but the response was not increased above the level achieved by a single antigen.

Lymphocyte activation by specific antigen could be inhibited by antibody. This is illustrated in Figure 3. First, it should be noted that the lymphocytes of a shark immunized to BGG reacted to 100 μg and 10 μg of the antigen, but not to 1,000 μg. Thus, excess antigen was inhibitory. This may be viewed as a form of in vitro tolerance. Antibody to BGG prevented lymphocyte response to the stimulatory doses of 10 μg and 100 μg and did not reverse the "paralyzing" effect of 1,000 μg.

Cellular immunity to rubella virus antigens was studied extensively in the snapper. Several preparations of virus produced in different cell substrates were used for immunization and for stimulation of lymphocytes in vitro in order to obviate the problem of evoking blastogenic responses to cellular antigens. This would have occurred if the same viral antigen, e.g., virus produced in rabbit kidney cells were used for both immunization and in vitro testing. Lymphocytes from peripheral blood, thymus, anterior kidney, and spleen were cultured in the presence of different concentrations of rubella
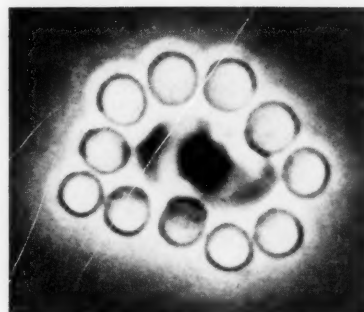
Figure 1.—Mixed hemagglutination reaction. In the center is a chicken red blood cell (RBC) and surrounding it are sheep RBC's. Shark natural antibody was mixed with the chicken RBC and subsequently washed to remove free antibody leaving only antibody bound to the chicken RBC. The attraction of sheep RBC is interpreted as indicating multispecificity of antibody.
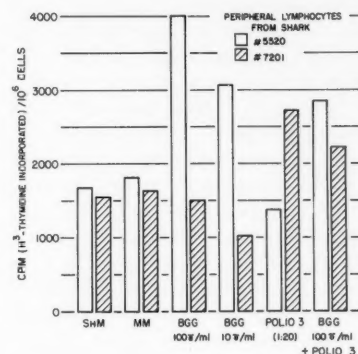
Figure 2.—Blastogenic response of immune shark lymphocytes to specific antigens. The test measures the incorporation of radioactive thymidine which denotes DNA synthesis evoked by the antigenic stimulation. ShM (shark growth medium) and MM (maintenance medium) are background controls indicating innate uptake of thymidine by unstimulated cells.

Figure 3.—Differences in response of shark lymphocytes to varying doses of BGG and the inhibition of the response by specific shark anti-BGG serum.

POOLED PERIPHERAL LYMPHOCYTES

Figure 4.—Blastogenic response of snapper peripheral blood lymphocytes to rubella virus. The hatched columns represent results of lymphocytes from immunized snappers. The solid columns represent the results with lymphocytes from nonimmunized snappers.

POOLED THYMOCYTES

Figure 5.—Blastogenic response of snapper thymocytes to rubella virus.

SPLEEN & PRONEPHROS CELLS

(POOL OF ORGANS FROM 2 SNAPPERS)

Figure 6.—Blastogenic response of snapper spleen and anterior kidney to rubella virus. Although in this experiment the spleens and anterior kidneys were pooled, in other experiments responses were observed with lymphocytes from separate organs. Furthermore, it has been possible to pool tissues from different snappers without causing allogenic responses.
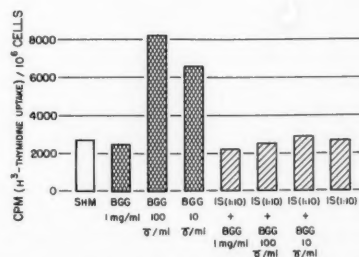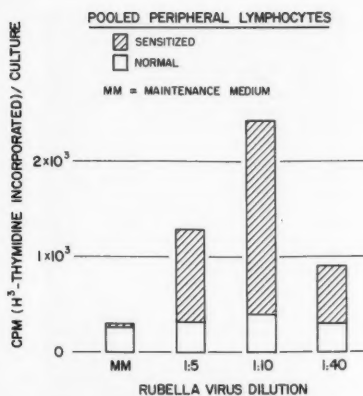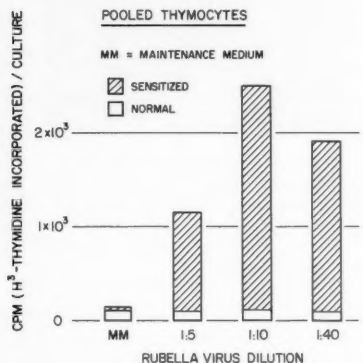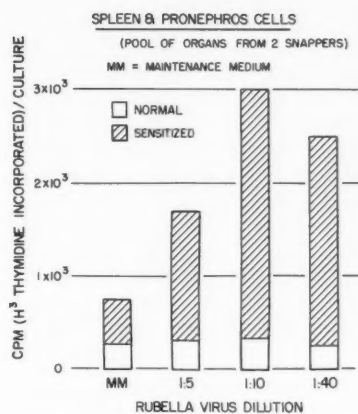
Table 1.—Effect of antirubella serum on the blastogenic reaction of sensitized snapper lymphocytes to rubella.

	Fish #8807		Fish #8808	
Stimulant	cpm[1]	S.I.[2]	cpm	S.I.
None	236	—	309	—
Culture fluid control	309	1.31	371	1.20
Rubella virus (1:10)	1,527	6.47	1,554	5.03
Ab[3] (1:10)	309	1.31	411	1.33
RV[4] (1:10)+Ab (1:10)	302	1.28	535	1.73
RV (1:10)+Ab (1:40)	503	2.13	572	1.85
RV (1:10)+Ab (1:160)	717	3.04	510	1.65
RV (1:10)+Ab (1:640)	850	3.60	658	2.13

[1]Counts per minute per culture; mean of triplicate cultures.
[2]Stimulation index = cpm in stimulated cultures/cpm in unstimulated cultures.
[3]Snapper antirubella serum, minimum titer haemagglutination inhibition 1:320.
[4]Rubella virus.

Table 2.—Effect of rabbit antirubella serum on stimulation of immune rabbit peripheral blood lymphocytes by rubella virus.

Stimulant	cpm ± SD[1]	S.I.[2]
None	459± 17	—
Control[3]	839± 24	1.83
RV (1:10)[4]	4,415±212	9.62
RV+Ab[5] (1:10)	1,648± 96	3.59
RV+Ab (1:20)	2,213±121	4.82
RV+Ab (1:40)	2,474±115	5.39
RV+Ab (1:80)	3,226±202	7.03
RV+Ab (1:160)	4,410±245	9.61
RV+Ab (1:320)	4,392±213	9.57
Ab (1:10)	495± 12	1.80
PHA[6]	8,996±510	19.60

[1]Counts per minute per culture; mean of triplicate cultures ± standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Rubella virus.
[5]Rabbit immune serum, heated at 56°C for 30 minutes before use.
[6]Phytohemagglutinin 0.01 ml (Lee and Sigel, 1974).

Table 3.—Differential effects of immune immunoglobulin-virus complexes on thymidine uptake by sensitized rabbit lymphocytes.

	Immune rabbit PBL		Normal rabbit PBL	
Stimulant	cpm±SD[1]	S.I.[2]	cpm±SD	S.I.
None	987± 28	—	1,056± 42	—
Control[3]	831± 49	0.84	1,014± 57	0.96
RV (1:40)[4]	13,626±952	13.81	1,122± 39	1.06
IgM[5]	999± 34	1.01	1,302± 62	1.23
IgG[6]	963± 24	0.97	1,350± 54	1.28
IgM+RV (1:40)	861± 62	0.87	1,188± 72	1.13
IgC+RV (1:40)	10,260±782	10.39	1,290± 45	1.22
PHA[7]	21,411±612	21.70	15,036±824	14.24

[1]Counts per minute per culture; mean of triplicate cultures ± standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Rubella virus.
[5]Pooled and concentrated immune 19S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 O.D.$_{280}$ units/ml. This IgM preparation had a haemagglutination inhibition titer of 20 VS rubella virus.
[6]Pooled and concentrated immune 7S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 O.D.$_{280}$ units/ml. This IgG preparation had a haemagglutination inhibition titer of 40 VS rubella virus.
[7]Phytohemagglutinin 0.01 ml (Lee and Sigel, 1974).

virus. The results are presented in Figures 4, 5, and 6. In all instances there were significant responses of lymphocytes from immunized fish to the antigen as measured by thymidine incorporation. One can observe in the figures an optimal dose effect. Lymphocytes from nonimmunized snappers showed no response to any concentration of virus. These blastogenic transformation reactions could be abrogated by the addition of snapper antibody directed against rubella virus. This is shown in Table 1.

Thus, in the experiments with sharks and with snappers, antibody was inhibitory to the lymphocyte transformation response. These results were of special interest in view of the fact that studies in other systems have failed to demonstrate inhibition of antigen induced lymphocyte responses by antibody (Rosenberg et al., 1972). In other experiments, the dominant antibody appeared to be IgG whereas the fish antibody was IgM, and this fact may have accounted for the difference. These findings have led us to inaugurate a project on the effect of different classes of antibody on the lymphocyte response to rubella virus.

DIFFERENCES IN THE EFFECTS OF IgM AND IgG ANTIBODIES ON BLASTOGENIC TRANSFORMATION AND THE EFFECT OF IMMUNE COMPLEXES

In order to measure differential effects of different classes of immunoglobulin on the blastogenic response to a viral antigen, experiments were undertaken with lymphocytes and different classes of immunoglobulin from rabbits immunized with rubella virus. These data have been published (Lee and Sigel, 1974), but are being reviewed now for the sake of completeness. In Table 2, findings are presented which illustrate the ability of rabbit immune serum to inhibit the response of rabbit immune lymphocytes to rubella virus. The differential effect of antirubella IgM and antirubella IgG is shown in Table 3. It is clear from this table that the IgM antibody suppressed

Table 4.—Effects of rubella virus-antibody complexes on the response of normal rabbit lymphocytes to PHA.

Stimulant	cpm $\pm$ SD[1]	S.I.[2]
None	721 $\pm$ 30	—
Control[3]	763 $\pm$ 35	1.05
PHA[4]	11,411 $\pm$ 590	15.82
RV (1:40)[5]	801 $\pm$ 8	1.11
RV + PHA	10,036 $\pm$ 113	13.91
IgM[6]	801 $\pm$ 18	1.11
IgM + RV	844 $\pm$ 42	1.17
[IgM + RV][7] + PHA	1,942 $\pm$ 150	2.69
IgG[8]	715 $\pm$ 20	0.99
IgG + RV	1,382 $\pm$ 38	1.92
[IgG + RV][7] + PHA	12,020 $\pm$ 342	16.67

[1]Counts per minute per culture; mean of triplicate cultures $\pm$ standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Phytohemagglutinin 0.01 ml.
[5]Rubella virus.
[6]Pooled and concentrated immune 19S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 O.D.$_{280}$ units/ml. This IgM preparation had a haemagglutination inhibition titer of 20 VS rubella virus.
[7]Mixtures of fractionated antibodies with virus were incubated at 37°C for 45 minutes after which they were added to lymphocyte cell cultures. The cultures were then put in a CO$_2$-enriched atmosphere at 37°C for 15 minutes before PHA was added.
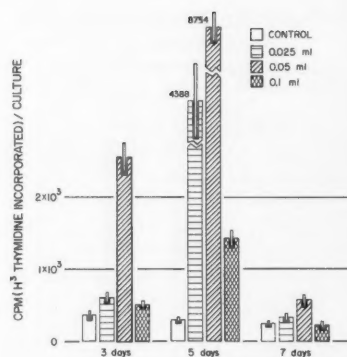[8]Pooled and concentrated immune 7S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 O.D.$_{280}$ units/ml. This IgG preparation had a haemagglutination inhibition titer of 40 VS rubella virus (Lee and Sigel, 1974).

Figure 7.—Blastogenic response of snapper peripheral blood lymphocytes to phytohemagglutinin (PHA). Control refers to lymphocyte cultures to which PHA was not added. The other columns show responses to different amounts of PHA as shown in the legend in the upper right.

Figure 8.—Separation of shark lymphocytes on Ficoll-Isopaque. Note the three layers of cells in the middle of the tube. An additional cell population of lymphocytes is located at the bottom of the tube.

the response whereas the IgG did not. These findings are in accord with the inhibitory effect of fish antibodies which belong to the IgM class.

In an attempt to gain insight into the mechanism of inhibition of blastogenic transformation, we have performed experiments in which the virus was allowed to complex with specific antibody, IgG or IgM, the immune complex was added to lymphocytes, and their response to mitogen PHA was determined. The results are given in Table 4. It can be seen that the IgM-antigen complex blocked the response of lymphocytes to PHA; the IgG-antigen complexes exerted no inhibitory action. This suggests that IgM antibody may play a more dominant role in regulation of the immune response. This finding assumes special significance in view of the presence of natural IgM antibodies in fishes, notably in the sharks, and it may explain, at least in part, the failure of sharks to mount a secondary immune response.
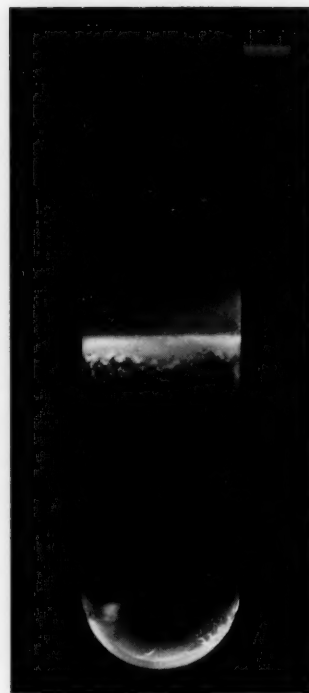
SUPPRESSOR CELLS

Still another mechanism of regulation is mediated by suppressor cells. These cells exert inhibitory effects against other lymphocytes upon activation by antigens, mitogens, or other factors. In higher vertebrates both T and B cells and probably monocytes are apparently capable of becoming suppressors (Gershon, 1974; Kirchner et al., 1974; Singhal et al., 1972). They dampen or stop immune responses in vivo and in vitro. It now appears from provisional findings that such cells also exist in fishes as evidenced by suppression of blastogenesis.

Snapper lymphocytes were shown to possess a relatively well-developed capability to react to plant mitogens. An experiment measuring the response of snapper peripheral blood lymphocytes to PHA is given in Figure 7. It can be seen that a fair response to PHA was evident in cultures at 3 days and a maximum response at 5 days. In contrast were the findings with lymphocytes from nurse sharks: here it was difficult to elicit a response to PHA, and the responses to Con A required high doses of mitogen. Although these responses are nonspecific as they do not reflect sensitivity to a specific antigen, they nevertheless are considered indicative of the overall immunologic status (or development) of the host. The findings would therefore imply some kind of deficit in the shark, presumably attributable to T cells. Further investigations were therefore conducted to elucidate this problem. Shark lymphocytes were separated on Ficoll-Isopaque[1]. This procedure has permitted the separation and recovery of at least three subpopulations of lymphocytes as illustrated in Figure 8. The individual bands or pellet of cells (bottom) were subjected to blastogenic transformation reactions with Con A and PHA. Results in Figure 9 show that all three subpopulations respond to Con A. In Figure 10 the results with PHA are shown. It should be noted that most of the subpopulations of shark lymphocytes did not react to PHA, but a reaction was obtained with the bottom cells. In one experiment (not shown) it was possible to inhibit the response of the bottom cells to PHA by the addition of interphase or top cells.

We conclude provisionally that sharks possess PHA responsive cells, and also suppressor cells, which are

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[1]Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

capable of inhibiting a function(s) of the responsive cells.

SUMMARY AND COMMENTS

1. The blastogenic transformation reaction is a useful indicator of immunity in fish. The ability of lymphocytes of sharks and bony fishes to react to specific antigens attests to a form differentiation observed in more advanced species, i.e., mammals. While specialized classes and subclasses of lymphocytes have been recognized in mammals, our present state of knowledge does not permit distinction in fish of true T or B cells and certainly not the subsets of T and B which perform different functions in mammals.

2. The blastogenic transformation reaction has demonstrated three modes of immunologic regulation: lymphocyte responses can be inhibited by IgM antibody, by IgM antibody-antigen complexes, and by suppressor cells.

3. Certain fishes possess natural antibodies with broad polyspecificity. These IgM immunoglobulins may have

a tremendous survival value for the sharks. Yet, the same antibodies may be responsible for immunologic amnesia and perhaps for diminution in cell-mediated immunity in the shark.

More information is urgently needed on these and other matters for the sake of knowledge about the intelligent approaches to immunization of fish (and avoidance of disastrous effects) and for the sake of furthering our understanding of immunologic developments and functions as they relate to other animals, including man.

ACKNOWLEDGMENT

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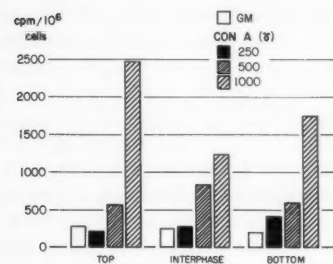

Figure 9.—Blastogenic response of shark lymphocytes to Concanavalin A (Con A) after separation on Ficoll-Isopaque.
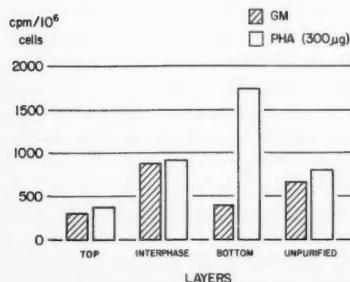

Figure 10.—Response of shark lymphocytes from the pellet (after separation on Ficoll-Isopaque) to Phytohemagglutinin.

Immunization of Fish With Bacterins of *Aeromonas salmonicida*

L. R. UDEY and J. L. FRYER

ABSTRACT—Highly virulent Aeromonas salmonicida *strains were shown to have an additional layer external to the normal cell wall, whereas avirulent strains lacked the layer. Strains possessing the additional layer were also found to aggregate and to adhere to cultured fish tissue cells. Consequently, the choice of* A. salmonicida *strains may play an important role in determining the nature of vaccine preparations.*

The route of administration of bacterins was also investigated. Three types of orally administered bacterins, produced using virulent strain SS-70, were not effective in conferring protection to juvenile coho salmon naturally challenged in a hatchery environment. Parenteral immunization with a whole cell bacterin in combination with Freund's Complete Adjuvant conferred a high degree of protection under the same challenge conditions. A possible explanation for the lack of protection in orally vaccinated fish was proposed.

Bacterial furunculosis has a long history in studies of fish diseases. The causative agent, *Aeromonas salmonicida,* was first isolated by Emmerich and Weibel (1894) in Germany. It was the first really well characterized bacterial fish disease organism and was isolated in this country in 1902 (Marsh, 1902). *Aeromonas salmonicida* has probably been the most studied of all bacterial fish disease organisms. Even though we now are able to control furunculosis disease fairly well with certain antibiotics (McCraw, 1952; Herman, 1968), there have been problems with drug resistant organisms.

The organism itself is a short, gram-negative, nonsporulating, nonmotile rod. It ferments selected carbohydrates anaerogenically, is cytochrome oxidase positive, and usually produces a characteristic melanin pigment from tyrosine. The disease is a generalized septicemia which can either be acute or chronic and causes necrosis and edematous lesions (Klontz et al., 1966). The disease has been found in practically every country in the world which raises fish (except Australia and New Zealand) and economically has probably been the most important fish disease agent.

Immunization of fish with *A. salmonicida* vaccines has been tried for many years. D. C. B. Duff, in the late 1930's and early 1940's introduced the idea of oral immunization in fish following attempts at human oral immunization with the enteric pathogens (Duff, 1942). His results were promising, but unfortunately, he was not able to carry on his experiments after the war. He prepared a chloroform-killed whole cell vaccine of a known virulent isolate and was able to show what looked like protection, although on a very small scale, in the laboratory.

G. W. Klontz pursued Duff's work at a later time and subsequently patented a vaccine which is termed Furunculosis Soluble Antigen (FSA). The FSA is a sonicated extract in which the cell wall remnants are centrifuged out and the soluble portion of the organism is precipitated with aluminum hydroxide and administered in the fishes' diet. In the laboratory, Klontz showed that this vaccination procedure was effective, but on subsequent field testing, its efficacy was questionable (Klontz, 1967, 1968).

Krantz (Krantz et al., 1964a, b) injected brook trout, *Salvelinus fontinalis,* and brown trout, *Salmo trutta,* with an *A. salmonicida* bacterin and found that those fish which were injected (especially in combination with Freund's adjuvant) were provided protection against laboratory challenge. This protection persisted even when the fish are severely stressed, e.g., at the time of spawning.

Results of previous experiments pointed to two problems in evaluating and developing vaccines: 1) There probably was not sufficient standardization of the challenge, and 2) not enough was known about the bacterium itself. The literature is somewhat confusing about the types of strains of this organism. This report is a discussion of the differences between aggregating and nonaggregating strains of *A. salmonicida* and how these differences might affect vaccines prepared from them.

AGGREGATING AND NONAGGREGATING STRAINS

A culture of the nonaggregating strain is shown on the left in Figure 1. On the right is the aggregating strain, and the bacteria can be seen settled into a small bottom zone. Figures 2A and B are scanning electron micrographs depicting clumped cells of the aggregating strain. We have shown this aggregation to be totally reversible and dependent upon divalent cations. In culture, the clumping can be extensive and the number of cells per aggregate ranges from 20 to 200.

Preliminary results suggested that these clumping strains appear to be

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Figure 1.—Cultures of nonaggregating (left) and aggregating (right) *Aeromonas salmonicida* strains.

more virulent than the nonclumping strains. In some cases, the use of a nonaggregating strain may have accounted for lack of immunization in previous vaccination attempts. This has been difficult to ascertain because the type of strain used as antigen was seldom reported.

We felt that the differences between the aggregating and nonaggregating strains might be significant enough to warrant a detailed investigation. To determine if there were differences between these strains, the cell wall structure was examined by transmission electron microscopy.

Figure 3 depicts a cell of the nonaggregating type of *A. salmonicida* with the characteristic gram-negative cell-wall structure. Illustrated are the rigid layer (R), the plasma membrane (PM), and the outer membrane area (OM). The structures labelled (B) are thought to be lipopolysaccharide extrusions. These appendages have also been recently reported on *Neisseria meningitidis*.

In contrast, we have found that the aggregating strains of *A. salmonicida* produce an additional layer (A) as shown in Figure 4. The additional layer itself in gram-negative bacteria is nothing new. The dark staining additional layer beyond the area where one would normally find the O antigen polysaccharides is similar to those described for *Acinetobacter* (Glauert and Thornley, 1969).

In an oblique section (Figure 5A) of
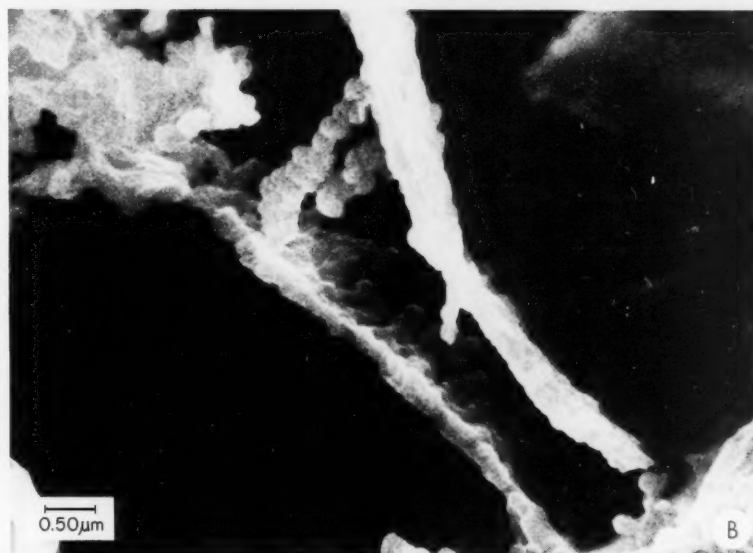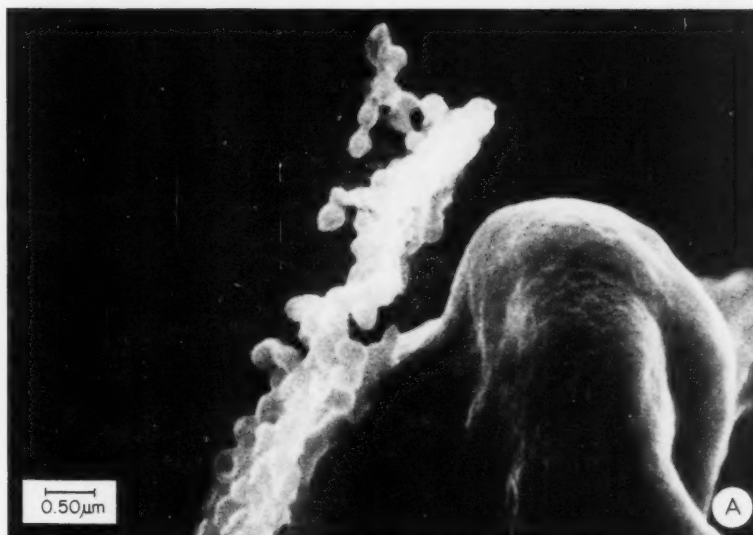
Figure 2.—Scanning electron micrographs of clumps formed by the aggregating strains of *Aeromonas salmonicida*.

an aggregating cell, the additional layer exhibits a periodic staining pattern which probably stems from the regular molecular configuration of the layer as demonstrated in additional layers of other bacteria. The additional layer is tightly bound to the outer membrane even when the cell is completely lysed, as seen in Figure 5B. This picture

shows the plasma membrane, rigid layer, and outer membrane with the additional layer still attached.

VIRULENCE OF STRAINS

We found that all of the strains from recent epizootics throughout the United States and British Columbia were of the aggregating type. We also found that
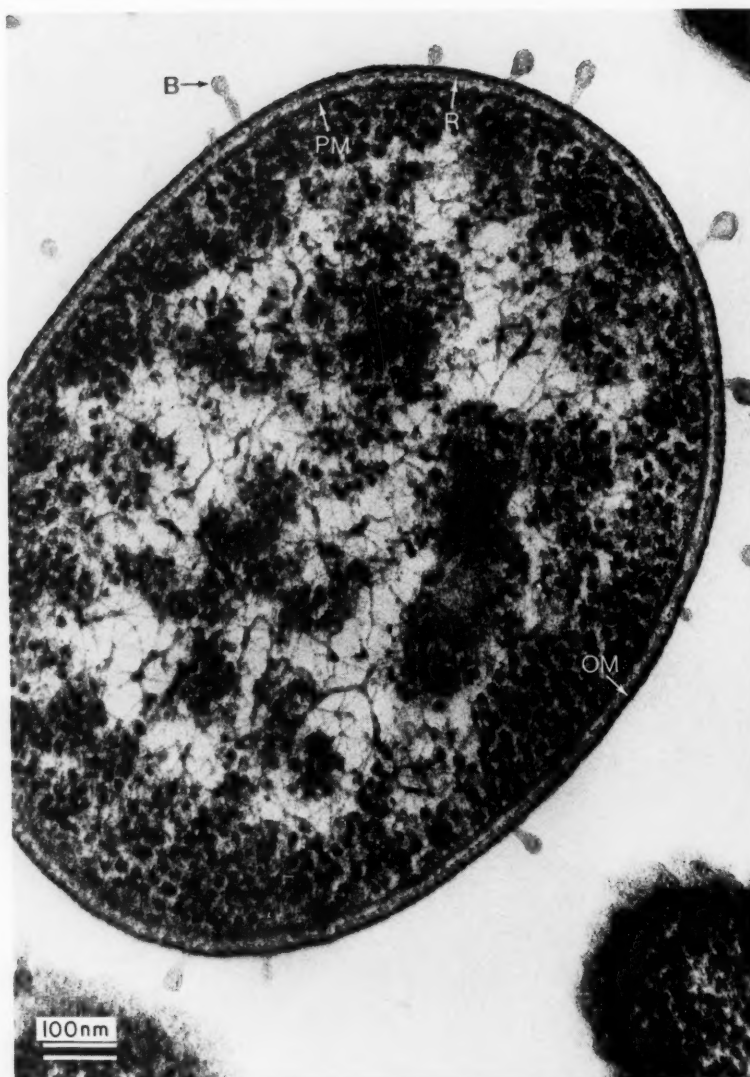
Figure 3.—Ultrathin section electron micrograph of a nonaggregating *Aeromonas salmonicida* cell. B = appendage, PM = plasma membrane, R = rigid or peptidoglycan layer, and OM = outer membrane.

maintained for several years on artificial media in our laboratory. Strain SIL-74 was obtained from a 1974 epizootic at the Siletz River Salmon Hatchery in Oregon. The final strain, 3.47 (EFDL), was also virulent in coho salmon and possessed both the additional layer and the ability to aggregate.

It is our conclusion that the additional layer is a requisite for virulence. The question still remains, however, whether this layer alone can confer virulence.

Additional layers have never been correlated with virulence before, nor have they been known to cause aggregation (Glauert, pers. commun.). The ability of bacteria to attach to tissue has been associated with virulence in infections caused by *Escherichia coli*, *Shigella dysenteriae*, and *Neisseria gonorrheae*. In these infections, however, the attachment of the bacterium is due to pili or to K-antigens. In preliminary experiments, the aggregating strains of *A. salmonicida* were found to adhere to human, rabbit, and fish white cells and to fish intestinal mucosa cells. This finding was interesting to us because it may be a mechanism by which the organism can deliver aggressins which it may possess. If the bacteria were attached to the tissue cell surface, aggressins could be secreted directly onto the cell without dilution. Klontz (1967, 1968) demonstrated a leukopenia in furunculosis infections, and suggested the presence of a leukocidin in *A. salmonicida*. It seems very likely that *A. salmonicida* could easily cause a leukopenia by attaching directly to leukocytes.

A model system was used to demonstrate that the aggregating strains have a marked affinity for tissue cells;

strains maintained for some time in culture collections were of the nonaggregating type. We compared the virulence of the two types to see whether there really was a correlation between aggregation, cell wall structure, and virulence (Table 1).

The ATCC 14174 culture is of the nonaggregating type, lacking the additional layer. Isolate SS-70 is virulent, and aggregating, and it possesses the additional layer. Strain SS-70-Sm^d-REV is a streptomycin-sensitive revertant derived from a streptomycin-dependent mutant. It has lost its aggregating ability as well as the additional layer but otherwise appears identical to SS-70. The SIL-67 strain was

Table 1.—Correlation between aggregation, cell wall (CW) structure, and virulence in *Aeromonas salmonicida* strains.

Strain	Aggregates	Staining CW layers	Lethal dose LD$_{50}$ (CFU) (colony forming units)
ATCC 14174 (NCMB-833)	—	3	$> 1.0 \times 10^8$
SS-Sm^d-REV	—	3	$> 1.0 \times 10^8$
SIL-67	—	3	$> 1.0 \times 10^8$
SS-70	+	4	8.8×10^0
SIL-74	+	4	6.8×10^0
3.47 (EFDL)	+	4	8.2×10^3

Figure 4.—Ultrathin section electron micrograph of an aggregating *Aeromonas salmonicida* cell. B = appendage, PM = plasma membrane, R = rigid layer, OM = outer membrane, A = additional layer.

100nm

VACCINES AND RESULTS OF VACCINATION

We felt also that the additional layer may well be an important antigen and that its presence should be considered in vaccine preparation. The effects of vaccine preparation methods on the recovery of Kjeldahl nitrogen and organic carbon from two strains of *A. salmonicida* were examined (Table 2). Strain Sil-67 is nonaggregating, and SS-70 is of the aggregating type. The cell wall of SS-70 was not as easily lysed, and the recoveries were consistently higher in the first four preparations. Since the FSA vaccine relies on obtaining soluble material, SS-70 gave a substantially lower yield (and different composition) than strain Sil-67.

Three of the vaccines listed in Table 2 were evaluated in an immunization trial. The Formalin[1]-killed whole cell, Formalin-killed whole cell + Al (OH)$_3$, and FSA vaccines were administered in the diet at 500 μg vaccine-carbon/gram of diet. Additionally the Formalin-killed whole cell vaccine was injected with Freund's Complete Adjuvant (FCA) intraperitoneally. Each vaccine was evaluated in three groups of 500 fish, and an additional three groups served as unvaccinated controls. All groups were exposed to a natural epizootic at Siletz River Salmon Hatchery using effluent water from a pond containing fish with
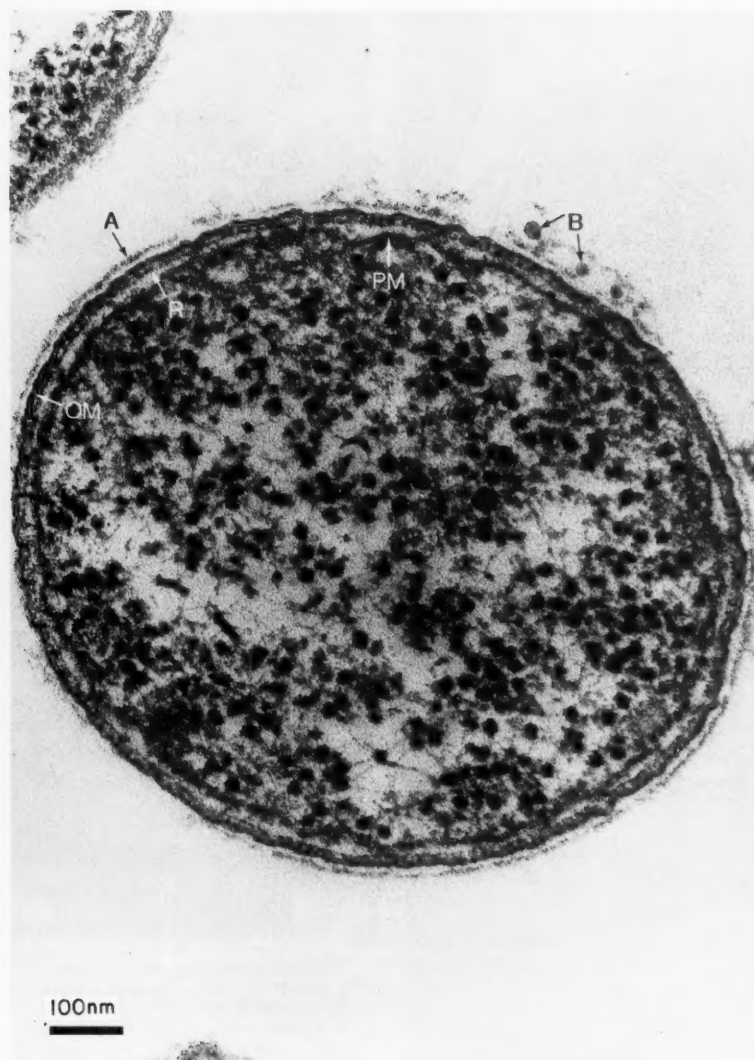
Table 2.—The effects of vaccine preparation on the recovery of nitrogen and carbon from two *Aeromonas salmonicida* bacterins.

Vaccine type	Vaccine strain			
	SIL-67		SS-70	
	%N	%C	%N	%C
Chloroform-killed whole cells	58.0	58.6	83.1	79.7
Chloroform-killed whole cells + Al(OH)$_3$	56.6	60.2	66.8	72.6
Formalin-killed whole cells	94.6	93.8	95.1	96.9
Formalin-killed whole cells + Al(OH)$_3$	77.6	78.0	96.8	99.1
FSA vaccine	60.1	65.3	33.0	31.4

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

the nonaggregating strains did not show this affinity. Figure 6A is a scanning electron micrograph of a chinook salmon embryo cell (CHSE-214) grown in monolayer. In the second picture in this series (Fig. 6B) the same cells were incubated with the nonaggregating strain SS-70-Sm^d-REV. It can be seen that the bacteria have little or no tendency to adhere to the CHSE cells. Strain SS-70, an aggregating strain, was incubated with CHSE cells and is shown in the third figure (Fig. 6C). The bacterial cells of this strain adhere markedly to the tissue cells; 30 times as many aggregating bacteria adhered to the tissue cells compared with the nonaggregating type.

Figure 5A.—An oblique section of an aggregating cell of *Aeromonas salmonicida* showing the periodic staining pattern of the additional layer (A). 5B. An ultrathin section through a lysed *Aeromonas salmonicida* cell of the aggregating type. Note that the additional layer (A) is still firmly bound to the outer membrane (OM).

Figure 6.—Scanning electron micrographs of: A. Normal (control) chinook salmon embryo (CHSE-214) cell attached to a glass surface. B. CHSE-214 tissue cells incubated 5 minutes with nonaggregating *Aeromonas salmonicida* cells. C. CHSE-214 tissue cells incubated 5 minutes with aggregating *Aeromonas salmonicida* cells. Clumps of bacteria can be seen attached to the tissue cells.

active furunculosis. Table 3 is a summary of the results of this experiment. We were able to show that none of the oral vaccines provided any protection from furunculosis. Fish injected with the bacterin possessed a high level of protection which resulted in significantly reduced mortality ($P < 0.01$), however.

This experiment was disappointing to say the least. I would like to comment briefly on a possible explanation for the lack of success using oral vaccination with *A. salmonicida*.

An experiment was conducted in which groups of fish were: 1) Untreated, 2) fed strain SS-70, 3) injected with FCA and SS-70, and 4) fed and injected with SS-70. The injected groups were inoculated on the same day as feeding began. Vaccine was fed for

45 days, and all groups were bled 63 and 93 days after the end of vaccine feeding. Fish which received only oral vaccine exhibited no significant increase in humoral agglutinins (Table 4). The group, which only received the injection of vaccine, gave agglutinin titers of 1:2,048.

Interestingly, the group which was both injected and fed vaccine had suppressed humoral antibody response. This trend was maintained even 93 days after the end of vaccine feeding. This phenomena may be a case of immune suppression. Much additional work needs to be done in this area before an effective oral *A. salmonicida* vaccine can be expected.
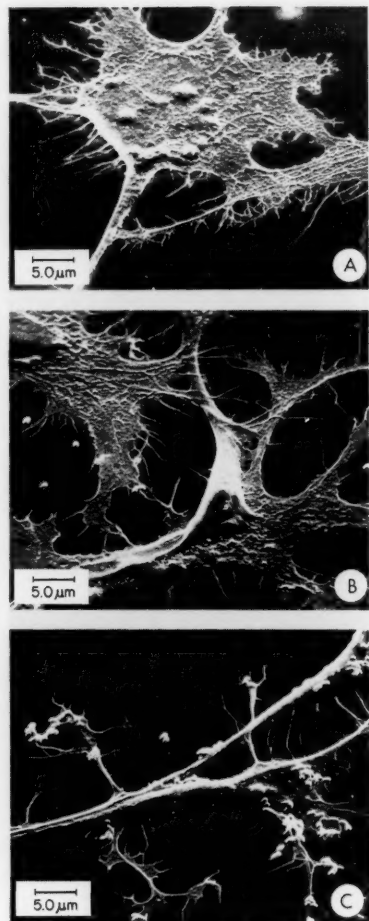
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Table 3.—Results of oral and parenteral vaccination of juvenile coho salmon with *Aeromonas salmonicida*.

Vaccine	Mortality (%) (± S.E.)	*A. salmonicida* mortality (%) (± S.E.)	Recovery rate (%) (± S.E.)
Formalin-killed whole cells (per. os.)	10.2 ± 1.4	8.6 ± 1.7	83.8 ± 2.2
Formalin-killed whole cells + Al(OH)₃ (per. os.)	12.8 ± 3.0	11.0 ± 2.4	85.8 ± 1.3
FSA vaccine (per. os.)	9.4 ± 1.4	7.5 ± 0.9	88.4 ± 0.4
Formalin-killed whole cells + FCA (i.p.)	3.5 ± 0.3	1.9 ± 0.6**	55.4 ± 10.7
Unvaccinated	12.4 ± 0.8	9.9 ± 0.2	78.7 ± 3.8

**Significant at $P < 0.01$.

Table 4.—Antibody response of juvenile coho salmon to *Aeromonas salmonicida* bacterins given orally and/or parenterally.

Treatment	Days after initiation	Days after end of feeding	Reciprocal agglutination titer
Untreated	0	—	16
Untreated	108	—	16
Untreated	138	—	32
Fed	0	—	16
Fed	108	63	32
Fed	138	93	64
Injected	0	—	32
Injected	108	—	2,048
Injected	138	—	1,024
Injected + fed	0	—	32
Injected + fed	108	63	128
Injected + fed	138	93	128

Immunization of Channel Catfish, *Ictalurus punctatus,* Against Two Bacterial Diseases

JOHN H. SCHACHTE, Jr.

ABSTRACT—*A field study was begun to compare the efficacy of three different routes of immunization of channel catfish,* Ictalurus punctatus, *intensively cultured in cages. A polyvalent bacterin against* Aeromonas hydrophila *and* Flexibacter columnaris *was administered by oral, injection, and immersion routes. Initial data indicated a humoral antibody response to all three routes. Thirty-day post-immunization agglutinin titers were as high as 1:1,280 in fish injected with bacterin, 1:160 in immersion-treated fish, and 1:20 in orally immunized fish. Gut mucosal extracts of identical fish had precipitin titers as high as 1:5,120 in immersed fish, 1:320 in orally immunized fish, and 1:160 in injected fish. Control titers were 1:80. No significant difference in protective immunity was detected. These data reflect initial results of a 2½-year study which will be reported in full at a later date.*

During recent years, cultural and nutritional studies with channel catfish, *Ictalurus punctatus*, at the Fisheries Research Unit at Auburn University have been plagued with bacterial disease problems. Epizootics of *Aeromonas hydrophila* and *Flexibacter columnaris* have been particularly troublesome in intensive culture techniques such as in pens and cages. These bacteria are also of importance in open pond culture, particularly under poor environmental conditions.

Experiments conducted at Auburn in 1972 indicated that the channel catfish would produce high circulating antibody titers when injected with a heat-killed bacterin. Additional studies also revealed that agglutinin titers as high as 1:160 could be induced following a single immersion treatment of bacterin.

METHODS OF TEST IMMUNIZATIONS

During the spring of 1974 a study was begun to test the possibility of immunizing channel catfish in cages against *A. hydrophila* and *F. columnaris*. The experiment was designed to test the level of protective immunity by observations of survival following natural infection with these bacteria in a pond. The experimental pond had a 5-year history of epizootics of these organisms in the cage culture of catfish. Differences in response of fish to three routes of administration of the bacterin were also measured by survival data, titering fish sera, and mucosal samples for circulating and secretory antibody. Twenty-four hundred channel catfish fingerlings were selected and divided into four groups of 600 fish, each group in a 730-liter tank. Three 600-fish groups were designated for bacterin administration by either injection, immersion, or oral routes, and the remaining group served as a control. A heat-inactivated polyvalent bacterin with adjuvant was prepared with *A. hydrophila* and *F. columnaris*. Injected fish received a single 0.2 ml intramuscular dose of bacterin with adjuvant while the immersion treatment consisted of the addition of bulk vaccine directly to the tank such that the final dilution was 1/126. Fish fed vaccine were administered treated feed every other day for a total of six treated rations; controls were held under identical conditions without bacterin administration. Following a suitable period for response to the bacterin, all fish were stocked randomly by treatment into 12 respective 0.75-m^3 cages anchored on a line in a 4.8-acre pond.

At 30 days postimmunization, a subsample was removed from each treatment group and the control group. Serum, surface mucus, and gut mucosal samples were collected and titered using microtiter techniques. Serum was titered against *A. hydrophila* and *F. columnaris* whole cell antigens, while mucosal extracts were tested against sonicates of the same organisms using a capillary tube precipitin technique.

RESULTS OF IMMUNIZATION

Following the 5-month experimental period, during which infections of both bacteria were diagnosed, the fish were counted for survival data. Sixty-eight percent of the controls survived, while 66 percent of the orally treated, 78 percent of the immersion treated, and 80 percent of the injected fish survived. Thirty day postimmunization agglutinin titers of injected fish were as high as 1:1,280, 1:160 in immersion-treated fish, and only 1:20 in feed-treated fish. Precipitin titers in gut mucosal extracts from the same fish were as high as 1:5,120 in immersed fish, 1:320 in feed-treated fish, and 1:160 in injected fish. Controls had a titer of 1:80 which was considered to be a result of exposure prior to the experiment. Statistical

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analysis of survival data revealed no significant differences ($P \geqslant 0.05$) between treatment groups and the control group or among treatment groups. This was thought to be a result of a high degree of variation encountered in the cages. However, percentage values seem to indicate that the immersion and injection routes might give significant results with further studies. The fact that there is a circulating and secretory antibody response to the immersion route seems to indicate that further refinement of this technique may result in an effective immunization method which will eliminate individual handling of each fish.

ACKNOWLEDGMENTS

Immunization of Salmonids for Control of Vibriosis

J. L. FRYER, J. S. ROHOVEC, and R. L. GARRISON

ABSTRACT—Experimental work done with the immunization of chinook salmon, Oncorhynchus tshawytscha, and coho salmon, O. kisutch, against Vibrio anguillarum is described. Experiments include: 1) A comparison of the efficacy of oral and injected bacterins; 2) a study of the protection provided by three selected oral bacterin concentrations; 3) an investigation of three selected periods of bacterin administration; and 4) a study of the effects of diverse water temperatures on oral immunization. Studies with the V. anguillarum bacterin indicate that under experimental conditions fish can be immunized against vibriosis.

Work on fish pathology at Oregon State University has included the development of several experimental immunogens including those of *Flexibacter columnaris*, *Aeromonas salmonicida*, infectious hematopoietic necrosis virus, and *Vibrio anguillarum*. Experiments with these bacterins and vaccines have been directed toward studying the immune response in Pacific salmon (genus *Oncorhynchus*) and steelhead trout, *Salmo gairdneri*. The primary objective of this research has been potential immunization of these animals for control of infectious diseases which have affected large-scale fish culturing attempts. Efficacious bacterins have been reported for the control of vibriosis in salmonids (Hayashi et al., 1964; Fryer et al., 1972). To date, the most successful immunogen tested in our laboratory has been prepared from cells of *V. anguillarum*.

One of the simplest methods for delivery of bacterins to large fish populations is by oral administration. Therefore, the major portion of this investiga-

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tion (begun in 1968) has involved developing oral bacterins and testing conditions under which they can be effectively used. In addition to oral immunogens, a parenteral administered bacterin was also studied.

METHODS

Preparation of Bacterins

The preparation of each antigen is outlined in Figure 1. The procedure for mass culturing of the bacterial cells was the same in the preparation of the oral bacterins. Ten ml of brain heart infusion (BHI) broth (Difco)[1] were inoculated with organisms from a stock culture of *V. anguillarum*. After 12 hours incubation at 25°C, 2 ml of this broth culture were used to inoculate two separate 1-liter quantities of broth which were also incubated 12 hours at 25°C. These two separate liters of culture were then used as the inoculum for 30 liters of medium which had been sterilized in a fermentor. After an incubation period of 10-12 hours at 28°C, 500 ml of a 20 percent dextrose solution was added and the culture allowed to incubate an additional 10-12 hours.

For the preparation of the wet-

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

packed, whole cell bacterin, the bacterial cells were killed in the fermentor by the addition of 100 ml Formalin solution directly into the 30 liter broth culture. The cells were harvested after 1 hour, frozen, and stored at −26°C.

To prepare lyophilized whole cells, the cells were harvested by high speed, continuous-flow centrifugation. Two hundred fifty grams of the harvested cells were then resuspended in 1 liter of a saline solution containing 0.3 percent Formalin and mixed for 24 hours at 25°C. The cells were then lyophilized.

Cells were also prepared for parenteral administration. Because smaller quantities of the bacterin were needed for this type of immunization, cells were cultured on BHI agar surfaces. This was done by preparing agar slants in 8-ounce prescription bottles which were inoculated with 1 ml of a 12 hour culture of the desired organism. After incubation (24 hours at 25°C), the cells were removed from the agar surface with 0.3 percent Formalin-saline solution. Bacterial cells remained in this solution for 1 hour and were then washed three times by centrifugation in phosphate buffered saline.

Bacterin Administration and Challenge

The method for oral vaccination followed a similar procedure in all experiments conducted. Fish, either chinook salmon, *O. tshawytscha*, or coho salmon, *O. kisutch*, were fed a diet containing the bacterin incorporated at a selected level per gram of ration. Diets used in this study were either Oregon Moist Pellets (OMP) (Hublou, 1963) or Oregon Test Diet (OTD) (Lee et al., 1967). This diet was administered to fish held at a freshwater facility supplied with pathogen-free well water at an ambient temperature of 12°C. It was also possible to vary the water temperature in experimental aquariums from 4° to 23°C.

Efficacy of the bacterins was tested by exposing experimental fish to a natural challenge of *V. anguillarum* in a saltwater rearing impoundment at Lint Slough on the Oregon coast. Groups of animals were held in 1-m diameter fiberglass tanks which were furnished

with saltwater. *Vibrio anguillarum* is endemic at this rearing impoundment and reaches epizootic proportions in the warmer months of the year when the water temperature rises to 12°C or above (Cisar and Fryer, 1969).

A necropsy was performed on all experimental animals which died during the challenge. The animals were examined for gross pathological symptoms, dissected aseptically, and bacteriological cultures were then prepared from kidney tissue on BHI agar. After incubation, the plates were examined for typical colonies of *V. anguillarum*. Presumptive tests included microscopic examination using the gram reaction, and morphology and motility by means of phase contrast microscopy. Recovery of *V. anguillarum* was confirmed with rapid slide agglutination tests using the suspected isolates as antigens and *V. anguillarum* antiserum which had been prepared in rabbits.

Comparison of Oral and Injected *Vibrio* Bacterins

An experiment was designed to determine whether parenteral administration of bacterin would provide protection to fish and to compare the efficacy of this method to oral immunization. Experimental groups of fall chinook salmon consisted of 200 fish in the orally vaccinated groups and 150 fish in the injected groups. The oral bacterin consisted of lyophilized whole cells incorporated into OTD at a concentration of 2 mg/g of ration. The fish received 100 g of diet/200 fish per day. The bacterin feeding period was 30 days followed by a 15 day postvaccination period. Fish which were injected intraperitoneally received approximately 2 × 10⁸ cells/animal in 0.1 ml of a Freund adjuvant-saline suspension and were vaccinated 50 days prior to the time they were challenged. Throughout this period in fresh water, these animals were maintained on a ration containing no bacterin. The orally and parenterally vaccinated fish were simultaneously exposed for 40 days to natural challenge with *V. anguillarum*. The mortality of these animals was compared to a similar group of fish which had received no bacterin.
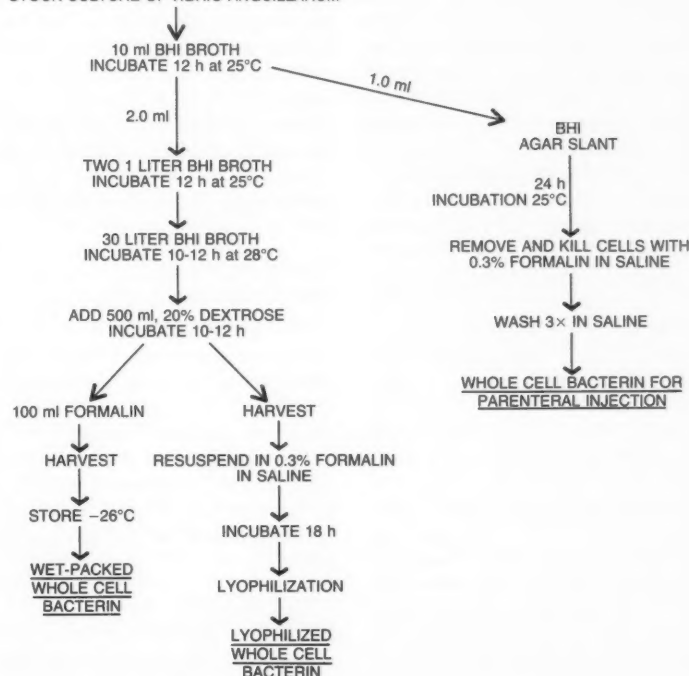
Figure 1.—Methods used for *Vibrio anguillarum* bacterin preparation.

Concentration of Oral Bacterin

Early experiments routinely employed either 5 or 10 mg of vaccine per gram of diet. These levels were arbitrarily selected and no comparative information was available. In order to gain some understanding of the concentration required to produce immunity with the orally administered bacterins, the following experiment was designed. Groups of 200 fall chinook salmon (mean weight 6.5 g) were fed wet-packed whole cell bacterin at levels of 2, 5, or 10 mg/g of OTD for 30 days. After this feeding period, the fish remained in fresh water for 15 days and were then challenged at Lint Slough for 30 days.

Length of Oral Bacterin Administration

There was also interest in the length of time that the vaccine containing ra-

tions should be administered to produce increased protection. The lyophilized whole cell bacterin was added to OMP at a level of 2 mg/g and fed to groups of 200 fall chinook salmon (mean weight 0.8 g). One group of animals received this diet for 15 days, a second for 30 days, and a third for 45 days. The vaccination periods were begun so that each of the three experimental groups received its last bacterin dose on the same day. All vaccinated fish and an unvaccinated control group were then maintained in fresh water for 15 days before being simultaneously challenged for 40 days at Lint Slough.

Effects of Temperature on Oral Immunization

Variations in water temperatures are encountered among hatchery locations and with the seasons of the year. It has been reported that agglutinating antibody production in poikilothermic

animals is slower at lower temperatures (Muroga and Egusa, 1969; Avtalion et al., 1973; Paterson and Fryer, 1974). Because all of the immunization experiments that have been previously described here had been conducted at water temperatures of 12°C, it was desirable to determine what effect diverse water temperatures had on the immune response of orally vaccinated animals.

Replicate groups of coho salmon (mean weight 3.3 g) were acclimated to each of seven temperatures: 3.9°, 6.7°, 9.5°, 12.2°, 15.0°, 17.8°, and 20.6°C. Acclimation of fish took place during a 1-week period at temperature increments of 3°C every 2 days. For 15 days these animals were fed a diet containing 2 mg of wet whole cell bacterin/gram of OMP. After this feeding period, each group of fish was held at its respective temperature for 1 week before being reacclimated to 12°C. The fish were challenged naturally at Lint Slough for 20 days and mortality was then compared to unvaccinated animals which had been held at 12°C throughout the freshwater phase of the experiment.

RESULTS

Comparison of Oral and Injected *Vibrio* Bacterins

Each group of fish vaccinated either orally or intraperitoneally showed little variation in mortality 40 days after challenge (Table 1). Injected fish had a 7 percent mortality, the orally vaccinated group 10 percent, and the control animals experienced an 80 percent loss. These results indicated that both methods of vaccine administration were effective for the control of vibriosis.

Prior to challenge, serum samples from fish of each vaccinated group were tested for the presence of agglutinating antibody. Animals which had been immunized parenterally had high antibody titers (1:640); however, fish which were orally immunized did not show detectable levels of agglutinating antibody. This indicated that different immune responses are stimulated by each of these two methods of vaccination. Similar observations have been made by Schaperclaus (1972). Although the mechanism of protection has

not been determined for orally immunized fish, cellular immunity or secretory antibody may be involved. Fletcher and White (1973) reported the presence of secretory antibody in the intestines of plaice, *Pleuronectes platessa* L., after oral administration of *V anguillarum* antigens. These experiments, however, have not been repeated using salmonids as experimental animals.

Concentrations of Oral Bacterins

The fish which were fed 2, 5, or 10 mg of bacterin/gram of ration experienced mortalities (due to vibriosis) of 25, 19, and 18 percent, respectively, 30 days after challenge. Sixty-six percent of the unimmunized fish died of vibriosis (Table 2). Results indicated that fish can be protected with a bacterin concentration of as low as 2 mg/g of ration.

Length of Oral Bacterin Administration

When the length of the vaccination period was varied, it appeared that increasing the time of administration to more than 15 days did not appreciably increase the protection provided these experimental animals (Table 3). Fish vaccinated for 15, 30, and 45 days experienced mortalities of 12, 13, and 16 percent, respectively, 40 days after challenge, while the unvaccinated control group had an 84 percent mortality.

Effects of Temperature on Oral Immunization

The results of this experiment indicate that diverse water temperatures do not preclude oral immunization with bacterins prepared for the control of *V. anguillarum*. All immunized groups had mortalities of less than 5 percent, 20 days after challenge. The control animals experienced losses as high as 83 percent (Table 4).

DISCUSSION

Throughout our research with the *V. anguillarum* immunogens, the efficacy of both oral and injected bacterins has appeared feasible. Since these studies indicate that under experimental conditions fish can be immunized against

Table 1.—Comparison of the efficacy of parenteral and oral administration of bacterin for the control of vibriosis in fall chinook salmon.

Method of bacterin administration	No. of fish/ group[1]	Total no. of deaths[2]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
Fed[3]	200	26	19	10
Injected intraperitoneally[4]	150	13	10	7
Unvaccinated control	200	172	160	80

[1]Mean weight 23.0 g/fish.
[2]After 40 days natural challenge to *Vibrio anguillarum* in saltwater.
[3]Bacterin was dry, whole cells incorporated into Oregon test diet and fed at a level of 2 mg of vaccine/gram of diet for 30 days.
[4]Bacterin was 0.1 ml of a Freund's adjuvant-saline suspension containing 2×10^8 cells.

Table 2.—Efficacy of selected bacterin concentrations for control of vibriosis in fall chinook salmon.

Bacterin concentration administered[1]	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
2	200	55	50	25
5	200	42	37	19
10	200	38	36	18
Unvaccinated control	200	139	132	66

[1]Milligrams of bacterin/gram of Oregon test diet fed for 15 days.
[2]Mean weight 6.5 g/fish.
[3]After 30 days natural challenge to *Vibrio anguillarum* in saltwater.

Table 3.—Efficacy of selected vaccination periods for control of vibriosis in fall chinook salmon.

Vaccination period[1] (days)	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
15	200	30	21	12
30	200	30	25	13
45	200	41	32	16
Unvaccinated control	200	170	168	84

[1]Bacterin fed at a level of 2 mg of lyophilized whole cell bacterin/gram of Oregon moist pellets.
[2]Mean weight 0.8 g/fish.
[3]After 40 days natural challenge to *Vibrio anguillarum* in saltwater.

vibriosis, the application of these techniques to aquaculture seems possible. However, to our knowledge, no definitive production trial has been conducted, and at the present time these products do not exist in a licensed form available to the aquaculture industry. We believe that before the pharmaceutical industry begins to prepare immunogens for licensing and use by fish culturists, seven major problems must be considered.

Table 4.—Efficacy of oral immunization of coho salmon held at selected water temperatures.

Temper-ature[1] (°C)	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
3.9	75	2	1	1
	100	13	5	5
6.7	95	4	2	2
	60	4	1	2
9.5	97	0	0	0
	100	6	2	2
12.2	90	2	0	0
	80	6	0	0
15.0	96	1	1	1
	100	0	0	0
17.8	97	0	0	0
	86	1	0	0
20.6	99	0	0	0
	87	0	0	0
Unvac-cinated control	100	72	72	72
	100	86	83	83

[1]Vaccinated with 5 mg of vaccine/gram of Oregon moist pellets for 15 days followed by a 7-day acclimating period.
[2]Mean weight 3.3 g/fish.
[3]After 20 days natural challenge to *Vibrio anguillarum* in saltwater.

1) There appear to be difficulties in moving from controlled laboratory experiments with 200-500 fish to full-scale production. Experimental results may vary with large numbers of fish in the production situation, and what is possible experimentally may not be directly applicable to aquaculture as it now exists.

2) Serotyping of fish pathogens is in its infancy, and the possibility of large numbers of serotypes of these pathogens could complicate the production of bacterin. Careful selection of bacteria or viruses for vaccine preparation must be made.

3) As techniques of immunology begin to be applied more extensively in aquaculture, there will be a real need to reemphasize the definition of immunity. There is a tendency to consider immunity as a shield built around the animals which makes them permanently invulnerable to a particular pathogen. Immunity is not permanent invulnerability. It is a relative state of insusceptibility brought on by one of three conditions: a) Having the disease and recovering; b) having a subclinical case of the disease; or c) being artificially immunized. The protection afforded by a particular immunogen is relative, and it can be overcome by any of several factors such as environmental stress, overwhelming presence of the pathogen, or the appearance of a different serotype. Therefore, progress in immunization of fish offers aquaculture increased protection from fish pathogens but not necessarily the eradication of disease.

4) Since environmental stress is a factor which influences susceptibility, and because fish are particularly vulnerable to these stresses, the impact of environmental alterations on the immune state needs further study. We have, on one occasion, seen an abrupt change in temperature produce sufficient stress in previously immune animals to cause them to become susceptible to the pathogen.

5) As work in both fish pathology and aquaculture continues, the development of reproducible artificial challenge systems will become imperative.

6) There is a need to develop methods for measuring immunity in fish other than direct exposure to the specific pathogen with subsequent evaluations based on death or survival.

7) Finally, novel methods for vaccine delivery should be explored. Although injection and oral administration of bacterins are capable of eliciting immune responses, each method poses inherent difficulties when dealing with large populations of fish.

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Vibriosis and Current Salmon Vaccination Procedures in Puget Sound, Washington

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ABSTRACT—The National Marine Fisheries Service aquaculture experiment station and several private salmon farms are successfully vaccinating fish against vibriosis via automatic syringe. Delivery systems other than injection are currently being researched and developed by private, governmental, and Sea Grant agencies. Evidently, there is some cross-protection offered by the original Vibrio anguillarum *isolate against a new vibrio species recently isolated at the Manchester, Wash., laboratory.*

VIBRIOSIS IN PEN-REARED SALMON

During the past 5 years the National Marine Fisheries Service (NMFS) has been rearing salmon and trout in saltwater at the NMFS Aquaculture Experiment Station on Puget Sound's Clam Bay near Manchester, Wash. (Novotny, 1975). It was obvious from the beginning that some form of preventive medicine other then chemotherapy was needed if the husbandry experimentation was to be successful. Vibriosis, the common fish disease caused by the marine pathogen *Vibrio anguillarum*, can cause high mortality among salmon in saltwater pens, particularly during the warmer summer months when water temperatures approach 15°C, and populations of fish in the pens are high.

A joint experimental pilot farm operated by the Union Carbide Corporation[1] and NMFS in 1971 involved experimental rearing of large numbers of coho salmon, *Oncorhynchus kisutch*, in saltwater pens. The results of this experiment clearly indicated that antibiotics were needed during vibrio disease outbreaks. After this pilot project proved successful in 1972, the Union Carbide Corporation decided to enter the salmon aquaculture business as Domsea Farms, Inc., and NMFS continued its research at the Manchester station.

VACCINATION PROCEDURES AND RESULTS

Preliminary studies indicated that a single injection of 2-4 mg of killed, wet-packed cells in physiological saline would be sufficient to protect coho salmon against the usual epizootics of vibriosis occurring in the central region of the Puget Sound area.

In the spring of 1973, the majority of experimental fish in the saltwater pens at Clam Bay were vaccinated intraperitoneally with heat-killed bacterin prepared from a *V. anguillarum* organism (Manchester isolate #775) that had been isolated regularly during the earlier net-pen experiments. Maximum agglutination titers of 1:32 were usually attained by 30 days postvaccination and declined over a 3-month period. Apparently, continued exposure to vibrio disease did not result in an anamnestic response in vaccinated fish. By November 1973, cumulative mor-

talities in our experimental fish were approximately 10 percent, which was decidedly more acceptable than the 60-90 percent losses that had been experienced before the use of chemical therapy or prophylaxis.

Domsea Farms had slightly over a million fish in saltwater pens in Clam Bay during the fall of 1973. Substantial losses began occurring in one of the larger net pens during November, and the moribund and dead fish were showing pronounced lesions typical of vibriosis.

At this time Domsea Farms had no pathology facilities and NMFS obtained several of the diseased specimens for a diagnostic workup. Vibrio organisms were isolated, but the growth characteristics were markedly different from those we had previously encountered. This organism was designated Manchester isolate #1669, and we began to further characterize it serologically and biochemically. Domsea Farms eventually lost approximately 90,000 ¼-pound coho salmon to this pathogen, primarily in one large net pen. Shortly after this, some of our experimental vaccinated coho were lost due to infections by this new pathogen.

No further losses to vibriosis occured with either our experimental fish or Domsea Farms' until the following summer growing season (1974) when nominal losses began to occur in our vaccinated fish.

Another new aquaculture facility, Pacific Ocean Farms, began growing about 300,000 coho salmon near Clam Bay in June 1974. All of these fish had been individually vaccinated by injection with #775 bacterin at least 2 weeks prior to saltwater entry. Our laboratory began to isolate the #1669 vibrio from these fish in August; the incidence increased as the agglutination titer to the #775 organism decreased. The losses continued at a low level until water temperatures dropped below 9°C. Total losses for the production season were

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[1]Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.
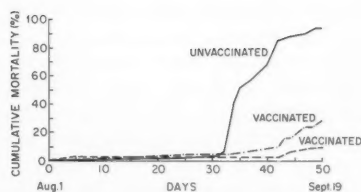
Figure 1.—Mortality of young-of-the-year (0-age) sockeye salmon smolts injected with #775 *V. anguillarum* vaccine. The fish were transferred from fresh water to net pens in the sea on 1 August 1974.

not substantial, even though another, as yet unidentified and unrelated, gall bladder lesion occurred in many of the fish.

On 1 August 1974, we transferred 450 sockeye salmon, *O. nerka,* smolts to saltwater net pens in Clam Bay. One pen contained 150 nonvaccinated control fish. Two pens contained equal lots of sockeye vaccinated with a heat-killed vibrio bacterin (isolate #775). Ninety-eight percent of the nonvaccinated sockeye died within the first 50 days, and only the #1669 type vibrio was isolated. Losses in the two vaccinated lots of fish were minimal at a total

of 32 fish (see Fig. 1), primarily due to #1669 type vibrio.

Serological studies with the two vibrio isolates and field observations of the three epizootics caused by the #1669 type organism led us to infer that there was some cross-protection with the #775 bacterin (Harrell et al, 1976).

There are now five aquaculture facilities in Puget Sound that are raising salmon in net pens (Mahnken, 1975). Estimated production for 1975 is 350 metric tons, of which about one-half will have been individually inoculated by injection with a bivalent #775-1669 vaccine. The remainder of the production will be receiving oral vaccines and/or chemotherapy as needed.

FUTURE DEVELOPMENTS

A new method of immunizing fish against vibriosis by immersion is currently under investigation by private

(commercial) and federally funded laboratories (D. F. Amend, pers. commun.).

During the summer of 1975 researchers at the Manchester station tested bivalent vibrio vaccines in field situations in cooperation with the Washington Department of Fisheries. The efficacy of several adjuvant preparations for injection with bacterins was also under investigation.

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Epizootiology of Channel Catfish Virus Disease

JOHN A. PLUMB

ABSTRACT—Channel catfish virus disease (CCVD) is a highly pathogenic herpesvirus disease of cultured channel catfish, Ictalurus punctatus, fry and fingerlings. The disease is confined to the southern United States, but occurs sporadically in other areas where young channel catfish are intensively cultured. Blue catfish, I. furcatus, have been experimentally infected with CCV, but other ictalurids appear to be refractory. The causative virus can be isolated only from fish showing clinical signs and morbidity. The clinical signs resemble those of other fish viral diseases. Histopathology is characterized by hemorrhage, necrosis, and edema in most tissues. It is possible to isolate CCV in brown bullhead, I. nebulosis, cells or cell cultures derived from channel catfish ovaries. The immunological response of channel catfish is discussed and theoretical control measures are suggested.

EPIZOOTIOLOGY

Channel catfish virus disease (CCVD) is a highly communicable viral disease of juvenile channel catfish, *Ictalurus punctatus*, in North America. The etiology of CCVD is a herpesvirus which results in hemorrhagia and edema in very young fish. It is primarily confined to cultured channel catfish populations in the southern United States where it occurs during the warmer months of June through September. Since the initial isolations (Fijan, 1968), CCVD has been confirmed in numerous epizootics in most southern states and several other localities where channel catfish are intensively cultured, including one Central American country.

Results of channel catfish virus (CCV) infections have varied from mild to catostrophic in different populations. The potential severity of the disease among susceptible channel catfish populations makes CCV a serious threat to culture systems already containing the virus, but its effect upon the catfish industry is not yet clear. Channel catfish virus has generally been found in fry or young-of-the-year fingerling channel catfish, although the virus was isolated from one yearling group of 18-cm fingerlings. Younger fish are much more susceptible, and in some cases 100 percent of infected fry have died involving individual lots of 8,000 to 3 million fish. In the fingerling stage (up to 5-10 cm and less than 5 months old) mortality may exceed 90 percent. However, size and age of fish alone do not determine the degree of mortality: environmental stresses such as low dissolved oxygen levels, high water temperature, crowding, and the stress of handling or transporting in-

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fected fish may influence the rate of mortality and may serve as triggering mechanisms for epizootics. Optimum water temperature for CCVD is above 25°C. Mortality may occur at 20°C, but below this temperature the effects of CCV are diminished. Secondary bacterial infections of *Aeromonas hydrophila* and *Flexibacter columnaris* also serve as synergistic forces in CCV infections and probably contribute to mortality.

TRANSMISSION

Channel catfish virus can be readily transmitted from infected fish to healthy fry or fingerlings by placing the healthy fish in water outfall from tanks or ponds holding infected stocks. The virus can also be readily transmitted to channel catfish by intramuscular or intraperitoneal injection, placing infected and noninfected fingerlings together in the same tank, swabbing the gills with virus, or by feeding infected materials. The possibility of vertical transmission from carrier adult fish to their offspring exists. There has been a tendency for epizootics to be associated with young from certain groups of broodfish, but the pattern of epizootic occurrences has been sufficiently erratic to shed some doubt on the theory of a definite adult carrier. In spite of numerous attempts, CCV has not been isolated from adult or subadult fish selected from a suspect carrier population.

Blue catfish, *Ictalurus furcatus*, can be experimentally infected by intraperitoneal injection. The virus could

not be transmitted horizontally from infected blue catfish fingerlings to non-infected fingerlings, or by feeding contaminated feed. White catfish, *Ictalurus catus,* and brown bullhead, *Ictalurus nebulosis,* fingerlings could not be experimentally infected.

Plumb et al. (1975) presented evidence that genetically different strains of channel catfish vary in their susceptibility to CCV (Fig. 1). Under control-led conditions the survival of experimentally infected 1- to 3-month old fingerlings ranged from 10 to 71 percent. Heterogenetic F_1 fingerlings had lower mortality than most homogenetic groups.

CLINICAL SIGNS AND PATHOGENESIS

The clinical signs of CCVD were described by Fijan et al. (1970) and Plumb (1971a). Typically infected fish are lethargic, with hemorrhages in the skin and at the base of fins, bilateral exophthalmia, distended abdomens, pale gills, and dark pigmentation. The body cavity may be filled with a clear yellowish fluid; the liver is generally pale; the kidney is enlarged and pale, but the spleen is deep red; the stomach and intestine are pale and void of food, but filled with yellow mucoid fluid. Some affected fish swim erratically and immediately prior to death may hang at the water surface in a head-up position, but this latter sympton may be caused by other diseases or environmental disorders, especially when very young fish are involved.

After experimental CCV infections of fingerlings, a viremia quickly develops (Plumb, 1971b; Plumb and Gaines, 1975). Virus was isolated from the kidney, liver, and intestine 24 hours after infection, but the kidney consistently produced higher titers than other organs (Fig. 2). Virus was also isolated from the blood, brain, and spleen (Fig. 3). Very little virus was isolated from the muscle. Virus titers reached peaks at 72 to 96 hours after infection and then subsided. Seven days after injection the virus levels in kidney, intestine, liver, and blood were 2-4 logs below the peak levels. However, virus titers in spleen and brain increased by 1-2 logs from day 6 to day 7 postinjection. The reason for these differences is not clear, but if infections were becoming dormant 6-7 days after infection, these two organs may be sites for development of latent infection.

Histologic findings of CCVD have been described primarily from experimentally infected fish (Wolf et al., 1972; Plumb et al., 1974; Plumb and Gaines, 1975; Majors et al., 1975). Internally, diseased fish exhibit: General hemorrhagia; liver epithelium and pancreatic tissue have focal necrosis; renal hematopoietic and excretory tissues are necrotic and edematous; while white and red pulp of the spleen are completely destroyed. Tissues of the diges-
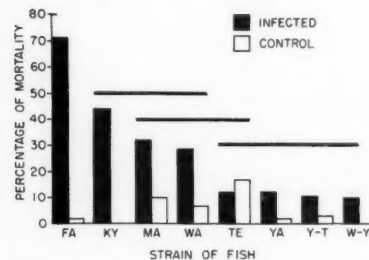
Figure 1.—Mortality of different strains of channel catfish fingerlings fed channel catfish virus. The strains infected were: Falcon (FA), Kentucky (KY), Marion (MA), Tennessee (TE), Warrior (WA), Yazoo (YA), Warrior-Yazoo (W-Y), Yazoo-Tennessee (Y-T). The means of any two strains that do not fall under the same line are significantly different (0.05). From Plumb et al., 1975.

Figure 2.—Postinjection titers of channel catfish virus in kidney, liver, and intestine of experimentally infected channel catfish fingerlings. From Plumb and Gaines, 1975.
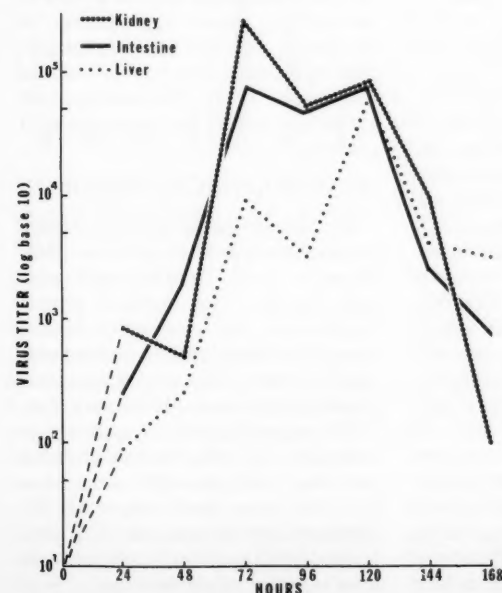
Figure 3.—Postinjection titers of channel catfish virus in blood, brain and spleen of experimentally infected channel catfish fingerlings. From Plumb and Gaines, 1975.
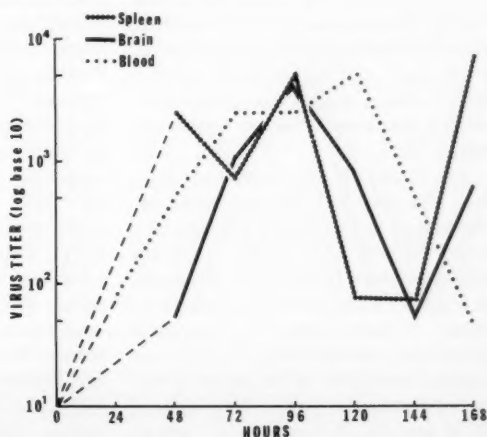
tive tract are edematous and hemorrhagic, and the mucosal layer of the intestine sloughs into the lumen. Skeletal muscle may have focal extravasation of blood, and brain tissue may be edematous. Histopathology of CCVD is very similar to that resulting from salmonid viruses in spite of their etiological differences (Yasutake, 1975).

Electron micrographs of tissues from experimentally infected fingerlings showed that CCV replicates in the nuclei of kidney, liver, and spleen cells (Plumb et al., 1974). Virus replication was characterized by the presence of intranuclear crystalline arrays and lamellar inclusions. Organ viral assay, and light and electron microscopy showed that CCVD is a systemic infection with generalized viremia. However, it appears that the kidney is the first and most severely affected organ.

MORPHOLOGY AND BIOPHYSICAL NATURE

Based on evidence presented by Wolf and Darlington (1971) CCV is an icosahedral herpesvirus with 172 capsomeres. The nucleocapsid measured 95-105 nm in diameter, but the enveloped particle has a diameter of 175-200 nm. The virus is ether, chloroform, and glycerol labile.

Channel catfish virus is very stable under frozen conditions in vitro and in vivo. Wolf (1973) reported that at −80°C in tissue culture media, no infectivity was lost after 9 months and at −20°C infectivity persisted for 4 months. Plumb et al. (1973) found that virus survived in infected fish frozen at −80° and −20°C for more than 6 months, for at least 14 days in iced fish, and for less than 3 days in specimens held at 22°C.

The virus is highly sensitive to drying. The virus is killed on dried concrete chips in less than 1 day and survives for only 1 to 2 days on nylon netting or glass. Infectivity is destroyed in less than 1 day in bottom muds from ponds. In water, however, survival is considerably longer (Fig. 4); CCV remained infective in organically enriched pond water for less than 4 days at 25°C and for 26 days at 4°C, but in
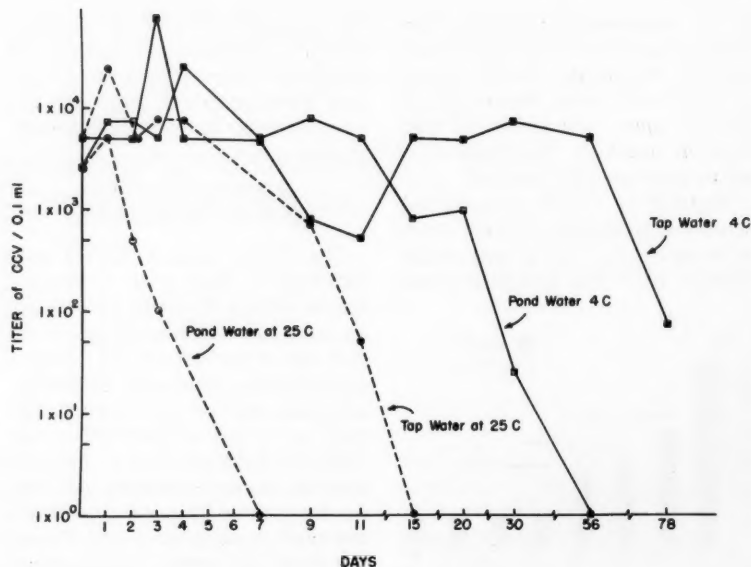
Figure 4.—Survival of channel catfish virus in pond and tap water at 4° and 25°C.

dechlorinated municipal water, infectivity was prolonged to approximately 11 days at 25°C and to more than 78 days at 4°C. Channel catfish virus does not survive well in water or on equipment at the temperature one would expect the disease to occur, especially if facilities and equipment are dried.

CELL CULTURE CHARACTERISTICS

The brown bullhead (BB) cell line (ATC-59) is the line of choice for CCV isolation and replication; however, cell culture derived from channel catfish ovaries are equally suitable. Wolf and Darlington (1971) failed to detect CCV replication in 15 other cell types including cells from other groups of fish, amphibia, birds, and mammals. Optimum replication temperature in BB cells is 30°−33°C, although replication will occur at 10°C (Wolf and Darlington, 1971). They found that at 30°C new virus was released 4 hours after cell culture inoculation, and cell associated virus reached a peak at 12 hours postinfection. The rate of virus replication in cell cultures is more rapid than in the fish as previously discussed (Plumb and Gaines, 1975). However, the cellular

ultrastructure changes are similar in vitro and in vivo.

Cytopathic effect of CCV in BB cell cultures is characterized by the development of syncytia resulting from coalescing of many infected cells and inclusion of their nuclei. Prior to syncytial formation, cells become pyknotic, basophilic, show aggregation of chromatin, and lamellar-like, intranuclear inclusion bodies appear (Wolf and Darlington, 1971). Karyorrhexis and karyolysis follow the appearance of syncytia.

IMMUNOLOGICAL RESPONSE

The immunological response of fish is temperature dependent (Bissett, 1948; Snieszko, 1970). Trout respond to antigens slowly at their optimum growth temperatures, but warm-water species, especially channel catfish, respond very rapidly under their higher optimum growth temperature. McGlamery et al. (1971) reported a positive antibody response in 2-4 weeks in channel catfish inoculated with stomatitis virus when the fish were held above 25°C. Heartwell (1975) detected CCV antibody at 28°C in channel catfish 1 week after injection of viable virus.

Figure 5.—Neutralization indices (log base 10) of 1-year-old channel catfish injected with channel catfish virus. Groups I and II were inoculated with infectious virus and Group I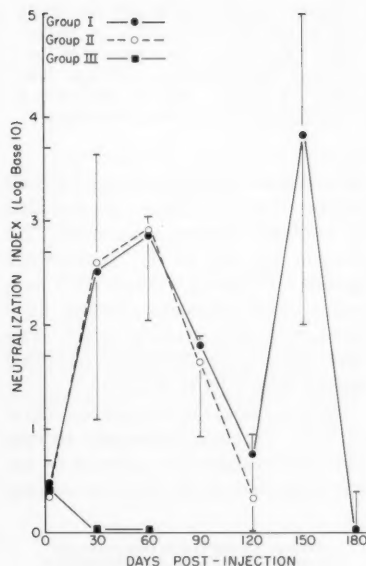II was inoculated with heat-killed virus. Group I was given a booster injection 120 days after the initial inoculation. From Plumb, 1973b.
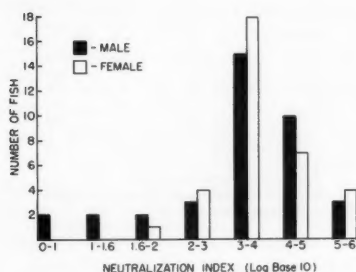

Figure 6.—Channel catfish virus (CCV) neutralization indices of sera from adult channel catfish with a history of CCV-diseased progeny.

Peak CCV specific antibody titers occur in adult channel catfish approximately 9 weeks after a single inoculum of virus (Fig. 5). A rapid anamnestic response will occur with a second injection after the initial peak titer, but it is weak and of short duration (Heartwell, 1975). Injection of heat-killed CCV elicits no immunological response. There is no information on the im-

munocompetence of fry or fingerling channel catfish to CCV.

The neutralization index (NI) was determined for sera from 67 adult channel catfish which had produced CCV diseased offspring for two consecutive years (Plumb, 1973b). The NI of these fish ranged from 1×10^1 to 1×10^5 with 76 percent of the NI values between 1×10^3 and 1×10^5 (Fig. 6). The NI of sera from a control group of adult channel catfish which had no history of CCV were all less than $1 \times 10^{1.25}$. Also, the NI from the CCV suspect fish remained at a high level throughout the year in contrast to the experimental immunological response reported by Plumb (1973b) and Heartwell (1975).

CONTROL

There is no known control of CCV; however, Plumb (1973a) reduced the water temperature from 28° to 18°C on infected fish 24 hours after infection and reduced the mortality from 95 to 24 percent. Reduction of temperature at the onset of clinical signs and death reduced mortality to 58-78 percent. In some instances where a fish farmer has cool water available and recognizes the signs of CCV, it may be possible to reduce the mortality by introducing cool water onto the population. However, the best means of combating CCVD is through avoidance of infected stocks. Detection of potential carriers is not possible through direct virus isolation. Broodfish previously associated with CCV epizootics had high levels of CCV specific antibody, therefore a serological approach may be possible to detect potential reservoirs of CCV.

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Serological Screening of Channel Catfish Virus

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ABSTRACT—Disease-free channel catfish, Ictalurus punctatus, *were used to study the pathogenesis of channel catfish virus disease and to determine a median lethal dose for exposed 30-day-old hatched fry. We were unable to establish an* LD_{50} *using fertile eggs and 30-day-old fry which were maintained under disease-free conditions. This data suggests that the high mortality seen in infected catfish under natural conditions is not due solely to the virus but results from concurrent infections and environmental conditions.*

In an effort to study in depth the pathogenesis of channel catfish virus (CCV) we attempted to: 1) Obtain disease-free fish, 2) develop the methodology for characterizing the infecting virus, and 3) assay the host response.

Our first need was a source of fish free of active or latent CCV and/or adventitious agents and diseases. We also wanted to raise these fish under laboratory conditions. The starting virus pool had to be characterized and purified. We obtained a pool of CCV from John Plumb[1] and used the plaque technique to clone the virus.

Our problems began when we tried to interpret our serological data and to establish disease and lethality patterns for the plaque-purified virus pool. We needed to choose: 1) A screening method to serologically define a population of fish with statistical validity, and 2) an assay technique to find latent infections and evidence of previous exposure to CCV.

SCREENING PROCEDURE

All serum samples were screened at a 1:8 dilution against a calculated challenge of 10 tissue culture infective doses ($TCID_{50}$) of CCV in a tube system using a minimum of three tubes per point. The design was based on our experience with a number of mammalian herpesviruses which we routinely screen. We have used this test for the last 3 years. As a part of this investigation, we screened a number of serum samples obtained from adult channel catfish, *Ictalurus punctatus,* from the Ft. Worth, Tex. area. These specimens were provided by J. P. McCraren[2] as part of a cooperative study. The acceptability of the results of the serological tests reported by our laboratory were questioned because our serum neutralization (SN) method was not in agreement with the 1974 recommendations of the CCVD Technical Procedures Committee.[3]

The criteria set up at the Denver meeting of the CCVD team for identifying a positive antibody response specified the use of a 1:100 serum dilution inoculated with 100-$TCID_{50}$. I think the test is not sensitive enough for most herpesviruses. We did not get that kind of antibody response normally, not even in fish that we hyperimmunized against CCV using virulent CCV in a water-in-oil adjuvant (Tween 80 - Arlacel-sterile mineral oil)[4]. The maximum antibody titers obtained ranged from 1:64 to 1:128.

Table 1 shows the results of our CCV antibody screening technique, and the data demonstrates the potential of the screening method. As can be seen in the

Table 1.—Comparison of tube serum neutralization screen test to log neutralization index test.

Fish serum	Tube screen	Interpretation[1]	LNI[2]
18	[3]2/3	−	0.5
19	0/3	+	4.0
24	0/3	+	5.4
26	0/3	+	3.0
27	2/3	−	0.0
32	0/3	+	3.0
41	0/3	+	3.0
43	0/3	+	5.0
44	0/3	+	[4]4.0
46	0/3	+	1.5
47	1/3	±	1.5
48	0/3	+	5.0
50	0/3	+	5.0
59	2/3	−	0.5
52	1/3	±	2.5
55	1/3	±	3.0

[1] − = no antibody; ± = suspicious; + = antibody.
[2] LNI = log neutralization index.
[3] Number of tubes with cytophathic effect (CPE) over total.
[4] Tested by the plaque reduction (90 percent) method.

table, all samples registered by the tube test as positive gave significant log neutralization indices (LNI) when tested in this manner. One sample, fish serum No. 44 tested only by the plaque reduction test neutralized 4 $\log_{10}$ of virus (90 percent of the plaque forming units present) as compared to the control titration. The test errs in the plusminus area where significant LNI were obtained in two of the three specimens recorded as suspicious.

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[1] Auburn University, Auburn, Ala.

[2] U.S. Fish and Wildlife Service, San Marcos, Tex.

[3] CCVD Technical Procedures Committee Meeting, Denver, Colo. 1974. (American Fisheries Society, Fish Health Section Meeting, U.S. Fish and Wildlife Service, 1975.)

[4] Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

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                    CATFISH EGGS
              ┌──────────┴──────────┐
             PBS                    CCV
              │                      │
          Hatched fry           Hatched fry
              │                      │
   Surviving 30 day old fish   Surviving 30 day old fish
         ┌────┴────┐              ┌────┴────┐
       CCV        PBS           CCV        PBS
     challenge              rechallenge
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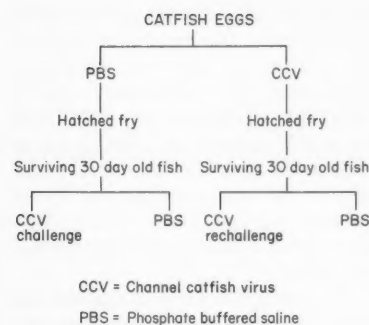CCV = Channel catfish virus

PBS = Phosphate buffered saline

Figure 1.—Schematic of egg and fish hatch and channel catfish virus (CCV) challenge. Challenge and control (phosphate buffered saline, PBS) exposures—immersion in beakers for 1 hour at 25°C.

Table 2.—Results of egg channel catfish virus challenge studies.

Group	Sample	Challenge dose[1]	Virus reisolation[2]	Fish sick or dead on hatch
1	Eggs	3.5 logs	No	All dead within 5 days
2	Eggs	2.5 logs	No	None + +[3]
3	Eggs	3.5 logs	No	None + +
Controls (1-3)	Eggs	PBS	No	None + +

[1]3.5 logs = 3,160-TCID$_{50}$/0.1ml; 2.5 logs = 316-TCID$_{50}$/0.1 ml; PBS = phosphate buffered saline—also used as virus diluent; TCID = tissue culture infected doses.
[2]Days sampled = 1 through 7.
[3]+ + = approximately 75 percent of all hatched fish survived. No difference between CCV-challenged eggs and PBS controls.

Table 3.—Results of channel catfish virus studies on 30-day-old catfish.

Group[1]	Sample[2]	Challenge dose	Virus	Fish sick or dead
2A	fish	PBS	no	none
2B	fish	2.5 logs	no	none
2C	fish	2.5 logs	yes[3]	yes
2D	fish	PBS	no	none
3A	fish	PBS	no	no
3B	fish	3.5 logs	yes[4]	yes
3C	fish	3.5 logs	yes[5]	yes
3D	fish	PBS	no	no

[1]A = control (PBS); B = egg control, 1st challenge; C = CCV egg, rechallenge; D = CCV egg, PBS control.
[2]Daily sample size = 8-10 fry.
[3]Days 4, 7, and 8 postinfection.
[4]Day 3 postinfection.
[5]Days 3, 4, and 6 postinfection.

ATTEMPTS TO ESTABLISH AN LD_{50}

Another problem was to attempt to establish an LD_{50} for cloned CCV in laboratory-raised fish. Establishment of an LD_{50} is important for the performance of back-challenge studies.

We screened a sizable number of fish from a hatchery with no known history of CCV. We found no evidence of CCV antibodies in the fish sampled. We obtained eggs from the hatchery and transported them to our laboratory tanks where equipment and hatching water were checked bacteriologically for freedom from contaminating organisms in an effort to establish and develop disease-free fish.

Lethality and pathogenesis were studied using two modes: 1) virulent CCV challenge of egg clutches, and 2) virulent CCV challenge of sac fry obtained from these egg clusters. A flow diagram for the CCV challenge is illustrated in Figure 1.

Three separate spawns of eggs were challenged with CCV using an immersion period of 1 hour. The first and third spawns were challenged with 1,000 $TCID_{50}$ of virus, and the second spawn was challenged with 100 $TCID_{50}$. After exposure to the virus, fluid was decanted, the eggs returned to a holding aquarium, and the batch was allowed to proceed. Samples of CCV and phosphate buffered saline (PBS) exposed eggs were collected immediately after exposure and at 24-hour intervals thereafter for 7 days.

The first phase of the study examined the susceptibility of fertilized eggs to CCV. The estimated number of eggs per group was 1,300 (CCV) and 1,300 (PBS) for group 1; 2,600 (CCV) and 3,600 (PBS) for group 2; and 2,400 (CCV) and 3,000 (PBS) for group 3. The number of hatched fish were: Group 1, 1,000 and 1,100; Group 2, 1,950 and 2,700; and Group 3, 1,800 and 2,250 with an average hatching survival of 75 percent.

Exposure or fertilized eggs to CCV by submerging them in either a 100 $TCID_{50}$/ml (group 2) or 1,000 $TCID_{50}$/ml (group 1 and 3) virus suspension did not result in the expression of overt disease, nor interfere with the resultant fry. The results of these challenge studies are shown in Table 2. No egg sample or resultant sac fry collected during the experiments yielded infectious virus on reisolation efforts, nor were we able to reisolate virus from the aquatic environment. All samples assayed were subpassaged three times before being considered negative for CCV.

In the second phase of the study the hatched fry (groups 2 and 3) were subdivided into four subsets and half of them challenged with the same dose of CCV as used for the egg clutch studies. For easy reference they were subdivided as follows: Progeny from the PBS egg control group were divided into a control subset (A) and inoculated subset (B) while progeny from the previously challenged group were divided likewise into a control subset (D) and into a rechallenged subset (C). The results are shown in Table 3.

Daily samples of randomly collected fry (8-12/day) were negative for virus in three of the four subsets in group 2. Virus was isolated, however, from apparently healthy fish in subset C collected on days 4 and 7.

Group III subset C registered virus on days 3, 4, and 6. The samples assayed on the third and fourth day sampling periods were positive for virus although no significant death pattern had occurred up to this period. Interestingly the death of 100 fry occurred in this group between days 4 and 5 postchallenge and more specifically between 7 and 16 hours postsampling on day 4. In addition, virus was also isolated from Group III subset B (day 3), however no overt clinical disease was evident nor were any virus-associated deaths observed. All virus isolates were subsequently identified as CCV by the SN test. However, the mortalities were not consistent and pathogenesis studies with these fish were discontinued.

Later, additional fish from the same supplier and the same age hatch were purchased and stabilized in the laboratory. We attempted to determine an LD_{50} for our cloned CCV by 1) immersion of the fish in log dilutions (1 through 7) of virus (1 hour at 27°C) and maintenance of these challenged fish at the same temperature; and 2) by intraperitoneal (IP) inoculation (0.05 ml) of log dilutions of the same virus pool.

LD$_{50}$ DETERMINATION RESULTS

We were unable to establish an LD$_{50}$. Our results can be summarized as follows.

Trial 1: immersion for 1 hour at 27°C (12/point).
A) Held 17 days with two deaths, one on day 6 and one day 7, both at 10^{-2}. Virus reisolated and identified by SN [(10^7/ml)—challenge titer].
B) Reexposed a second time to the same virus dilution, observed 10 days longer; no deaths resulted (titer 10^9/ml).
Trial 2: IP, 0.05 ml, 10^0 through 10^{-3}. All died within 24 hours, cause undetermined.
Trial 3: IP, 0.05 ml 10^{-5} through 10^{-8}.
A) Titer of pool 10$^{5.5}$/ml (12 point). No deaths in 7 days.
B) Reinoculated the same groups on day 8 using 10^{-2} through 10^{-5} dilutions. One death on day 6 at 10^{-3} dilution.
C) Fifteen days after intitial exposure. Flumethasone 0.5 mg (an anti-inflammatory adrenocortical steroid) was added to the 10^{-2} tank and to the PBS control tank. These were observed for 12 days. No disease. (Note: Each tank had 12 gallons of water when the corticosteroid was added.)

D) Challenge these fish with a one pass field isolate 29 days after initial exposure. Observed an additional 30 days with no death or illness seen. (Titer 10$^{6.5}$/ml.)
E) Blood was collected from caudal vein of these fish, pooled, and allowed to clot. Serum extracted from this blood was assayed for antibody to CCV. Pooled sera showed significant levels of antibody by the log neutralization index test.

DISCUSSION

Perhaps our challenge techniques have been insensitive. Immersion times of longer than 1 hour or injections of greater than 0.05 ml may precipitate a more consistent mortality. We found at this size and age any quantity of fluid in excess of 0.05 ml either just leaked out or, if great enough, the fish were blown apart from fluid pressure. Regardless, we were unable in any of our trials to establish an LD$_{50}$ with our pools of CCV.

Failure to establish an LD$_{50}$ may be attributed either to: A) Genetic resistance of the fish used; B) the fish may have had latent infections, thus creating a resistance to reinfection; C) modification of the virulence of cloned CCV; or D) high mortality in naturally infected catfish due to another infection rather than to CCV alone.

Genetic resistance in catfish had been

suggested earlier and this feature merits further study. We may have modified the virulence of our virus by plaque purification, but comparative studies with field isolates indicates little if any strain differences exist. The development of SN antibody confirms an exposure to the virus accompanied by an immune response albeit without clinical evidence of illness and a low mortality index.

We must define a way to establish the presence of latent infections in fish. My contention is that the high mortality seen in infected catfish under natural conditions is not due solely to CCV, but results from intercurrent infections plus environmental stresses. In many instances we have attempted to isolate virus from so-called outbreaks of CCV and have failed. We have never failed to isolate a number of other contaminating organisms.

ACKNOWLEDGMENTS

A Herpesvirus Disease of Green Sea Turtles in Aquaculture

HAROLD HAINES

ABSTRACT—Hatchling green sea turtles, Chelonia mydas, in aquaculture are affected, early in life, by a herpesvirus disease called gray-patch disease. Gray-patch disease is primarily a cutaneous disorder which may, in some animals, become severe enough to cause death. The cutaneous lesions typically occur in epizootic fashion and upwards of 60-95 percent of separate groups of hatchlings may be affected. There is no cure and the lesions generally resolve spontaneously in animals that do not die. The morbidity and mortality level is dependent upon the degree of stress which the animals undergo. Elevated water temperature appears to be the most important of several possible stress factors. An inactivated viral vaccine has been developed which holds promise for prevention.

For the past 9 years, there has been a serious attempt to commercially farm the green sea turtle, *Chelonia mydas,* on a large farm Cayman turtle farms (e.g., Mariculture, LTD.[1]), on the Island of Grand Cayman in the British West Indies. This farm is raising green sea turtles from hatchlings to marketable size in a total period of approximately 3 years. For the most part, the turtles are raised under stressful conditions of intensive crowding, high organic pollution, and fluctuating water temperatures, conditions which favor the outbreak of infectious diseases.

Over the past 4 years, we have studied some of these diseases to identify the etiologic agents and establish programs of prevention or control.

Table 1 lists the major diseases seen on the turtle farm and their presumed etiological agents, if known. By far the most intensively studied of these diseases has been an herpesvirus disease which affects the turtles during their first year of life. Before describing the herpesvirus disease, it is important to point out the nature of the farm facilities

and describe some of the farm operations which may contribute to individual outbreaks.

TURTLE FARM FACILITIES AND OPERATIONS

The commercial turtle farm is located on approximately 4 hectares (10 acres) of land. The holding facilities are composed of 170 concrete and plastic tanks and pens of varying capacities ranging from 454 liters to 340,650 liters (120 gallons to 90,000 gallons). At the same site, there are 7.57 million liter (2 million gallon), man-made breeding ponds which hold large breeding animals. A beach for egg-laying breeders, a slaughterhouse, a hatchery, a newborn tank area, and the stock farm are also located on the 4-hectare site, along with administrative facilities and a tourist shop. There is a pumping system which circulates approximately 9.46 million liters (2.5 million gallons) per hour of fresh seawater, drawn directly from the Caribbean, through the pens and tanks. The water is circulated through the farm, and allowed to empty into the ocean at an effluent canal approximately 400 m (0.25 mile) from the inflow pipes. At any given time, there are upwards of 50,000-100,000 green sea turtles of all ages on the farm. The stocking density is high: large numbers of turtles are housed in each tank, some of the larger tanks holding up to 2,000 turtles.

During daily operations, turtles are frequently moved from tank to tank, and various groups of animals may be mixed. Feeding is done on a daily basis, and uneaten food often settles to the bottom of pens or tanks where residue is removed by mechanical means.

The general operations of the farm and handling of the turtles appear to play an important role in disease production. In the past, wild turtle eggs were brought in from various beaches (Costa Rica, Surinam, and Ascension Island), and young turtles were hatched on the farm from these eggs. It is possible that some of the organisms currently found on the farm were incidentally transported with the eggs from various parts of the world. In addition, turtles derived from the different natural beaches were often mixed after hatching, a situation which may reinforce spread and maintenance of infectious organisms.

Today, a portion of the turtle eggs are still brought in from the wild, from Surinam only, and numbers of the young turtle stock are now obtained from eggs that have been laid on the farm by large breeders. It is projected that the turtle farm will become self-sufficient by 1980 and that all new

Table 1.—Diseases commonly seen in commercially raised green sea turtles.

Disease	Tissues affected	Causative agent
Gray-patch	Skin, shell	Herpesvirus
Floppy-flipper	Skeletal muscles	Virus? Clostridium?
L.E.T.	Lungs, eyes, throat, ear canals	Herpesvirus?
Coccidiosis	Gastrointestinal tract	Coccidia (*Caryospora chelonia*)
Skin lesions	Skin	Fungi, Bacteria

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hatchlings will come from farm-laid eggs. Since the breeding pond and the beach upon which nests are built are located at the same site as the stock farm, a situation exists in which disease organisms can spread to the breeders, and perhaps to the eggs, from stock animals, either by aerosol sprays produced from the circulating seawater, or perhaps by insects, wild birds, or rodents.

A young hatchling turtle is very small, only a few ounces in weight. In 3-3½ years, the baby turtles reach the size of approximately 45 kg (100 pounds); then they are usually slaughtered. There is generally a large mortality during the first year of life. Much of this loss is apparently caused by a herpesvirus infection to which we have given the name of gray-patch disease (Table 1).

GRAY-PATCH DISEASE

Clinical and Histological Picture

Details of the clinical and histological pictures of gray-patch disease, the discovery of a herpesvirus in the lesions, and the artificial transmission of the disease have been published (Rebell et al., 1975). Gray-patch disease occurs in two forms. One form is a pustular-like lesion which is found on the skin of the neck and flippers of young turtles (Fig. 1). The disease in this form is benign, and does not seem to harm the turtle. Generally, these lesions resolve spontaneously. The second form of the disease is manifested in a more extensive lesion in which obvious grayish patches are seen (Fig. 2). There may be spreading of these gray patches to large areas of the epidermal surface of the turtle, and in the most severely affected turtles, the complete epidermal surface may be covered. Often, these animals die from the disease.

The histological appearance of these lesions is typically one of a herpesvirus infection (Fig. 3) in which there are large cells containing nuclear inclusion bodies in the epidermis of the affected animals (Fig. 4). One receives the impression that if these animals were not living in an aquatic environment, the lesions would develop into distinct blisters, similar to those seen in the common herpesvirus infections of man,

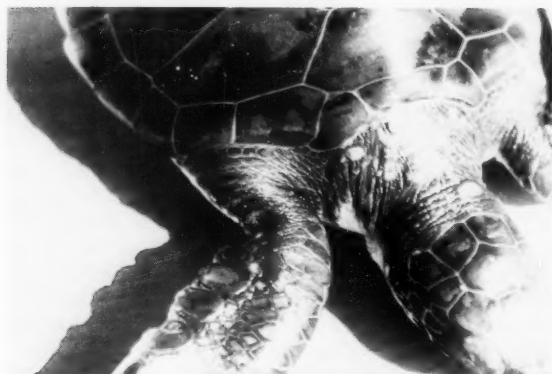Figure 1.—Gray-patch disease in young sea turtle showing "pustular" lesions on neck and flippers.
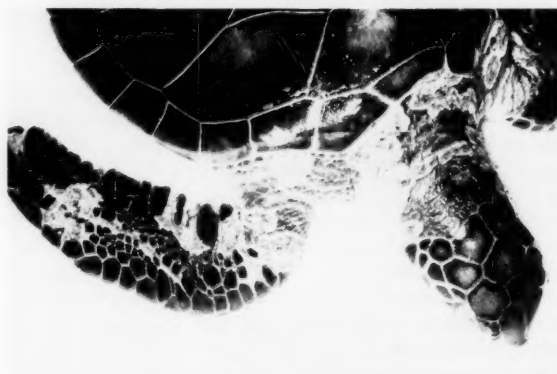

Figure 2.—Gray-patch disease in young green sea turtles showing spreading lesions on flippers and neck.
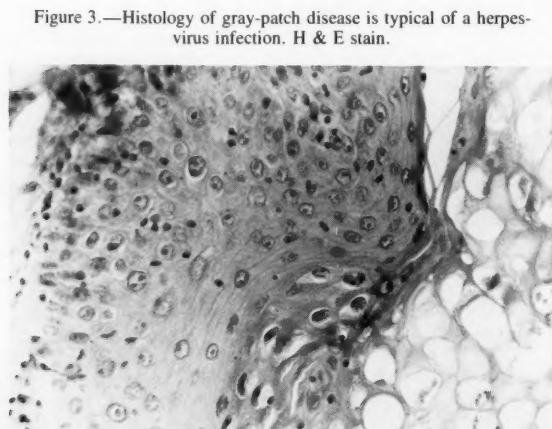
Figure 3.—Histology of gray-patch disease is typical of a herpesvirus infection. H & E stain.
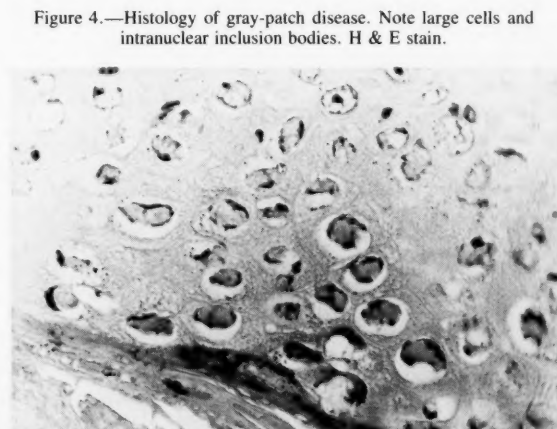
Figure 4.—Histology of gray-patch disease. Note large cells and intranuclear inclusion bodies. H & E stain.

such as herpes simplex or chicken pox. However, because of the aquatic environment, there is no epidermal roof to the gray-patch lesion, and any fluid which might form into a blister is quickly washed away. It is interesting to note that these lesions are truly epidermal in nature, and do not involve the dermal areas. No scars are formed on the skin of animals in which the lesions spontaneously resolve, unless as occasionally happens, the lesions become infected with bacteria or fungi which may invade the dermal area and deeper tissue.

Presence of Virus in the Lesions

Scrapings obtained from the gray-patch lesions, and examined by electron microscopy, show numerous herpes-type viruses. Figures 5 and 6 are electron photomicrographs which show the typical appearance of the gray-patch disease herpesvirus. Figure 6 is a higher magnification. As the electron photomicrograph illustrates, the viruses have a distinct capsid structure containing a nucleoid. The nucleocapsid is surrounded by an envelope. There also appear to be numerous empty capsids in lesion scrapings.

Because of the contaminated nature of the aquatic environment, bacteria also appear in gray-patch lesions. The bacterial flora of the lesions appears to change, depending upon the surrounding conditions, and we have never been able to show that a given strain of bacterium is a consistent inhabitant of the gray-patch lesions. Apparently, the lesions provide a suitable tissue for secondary infection and new bacteria come in as old ones are displaced. As mentioned, an occasional secondary bacterial infection does become so severe as to initiate deep scarring.

Transmission Studies

To prove that gray-patch disease is caused by the herpesvirus found in the lesions, we carried out transmission studies in which antibiotic-treated or filtered gray-patch lesion scrapings were scratch-inoculated into the epidermis of young, susceptible turtles. In 100 percent of the animals inoculated, the disease was produced but not in mock-infected controls. The new lesions produced by transmitted virus

Figure 5.—Electron micrograph of a herpes-type virus in scraping from gray-patch lesion.

Figure 6.—Electron micrograph at higher magnification of herpes-type virus shown in Figure 5.

Figure 7.—Scratch-inoculated flipper showing gray-patch lesion along scratch line.

Figure 8.—Mock-infected scratch-inoculated control flipper. Note absence of lesion.

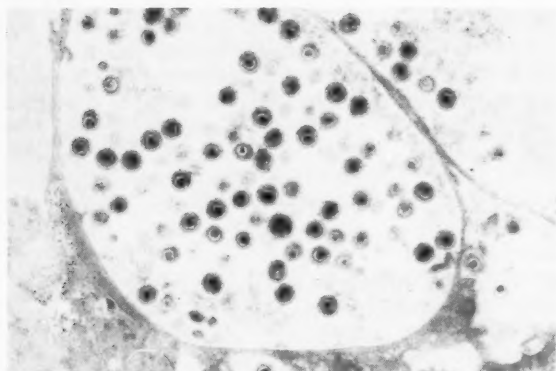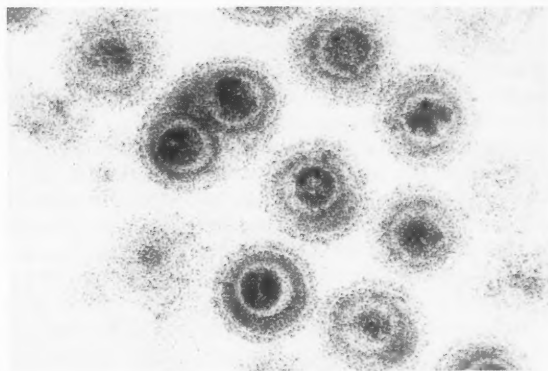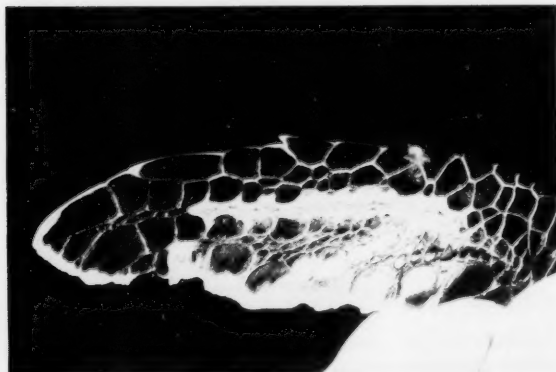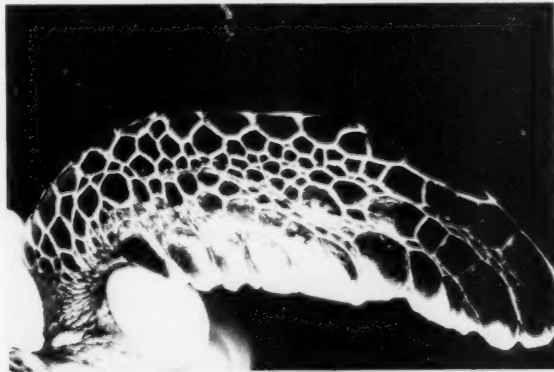also contained large numbers of the herpes-type virus, as determined by electron microscopy.

Figure 7 is a flipper of an animal which has had the virus transmitted to the epidermis by scratch-inoculation along a line. This figure clearly shows the gray-patch lesion along the scratch-line. Figure 8 is a control flipper which was mock scratch-inoculated. There is no lesion along the scratch-line.

Development of Natural Gray-Patch Disease

There is an interesting feature to the artificial transmission of the turtle herpesvirus. It appears that the susceptibility of the inoculated turtle depends upon its age. This age dependency apparently parallels the critical times in the life of the turtle at which they naturally acquire gray-patch disease.

The first critical time in the life of the turtle is around 2-6 weeks of age. Turtles of this age do not appear to acquire the typical skin lesions of gray-patch disease; however, there is a consistent level of mortality in this age group, usually about 20 percent. If 2-6-week-old turtles are scratch-inoculated with gray-patch disease virus, or are injected with the virus, 100 percent of them die within a short period of time. These animals die so quickly they do not have a chance to develop the typical skin lesions of gray-patch disease. The only obvious clinical sign is impacted fecal material in the lower intestinal tract. Interestingly, this is the same clinical sign that appears in the animals that die naturally during the first 2-6 weeks of life. This observation appears to indicate that the herpesvirus infection may cause a severe disease and mortality in some of the young turtles. Possibly the animals initially acquire the herpesvirus at this stage in their life.

The second critical time in the life of the turtle is from 8 to 15 weeks of age, when the majority of turtles that have survived develop gray-patch lesions in one of its two clinical forms. Some of these turtles die, but not nearly as many as in the initial peak of mortality during the first 2-6 weeks of life. It is during this 8- to 15-week period that typical spreading gray-patch disease appears.

It is also during this period of time that we are able to obtain large amounts of skin scrapings containing the herpesvirus. This material is useful for preparation of vaccines, for transmission studies, and for laboratory work with the virus.

Because the virus is present on the surface of the turtle, it is very possible that it is transmitted throughout the farm by water with new virus being shed constantly from infected turtles.

It appears that the severity of gray-patch lesions and also the mortality rate depend upon stress factors to which the turtles are subjected. For example, if the animals develop gray-patch disease in the summer, a season in which the water temperature of the tanks is high (around 30°C), the lesions appear to be very severe, and there is also a somewhat higher mortality rate. The opposite seems to be true in the colder winter months.

Turtles in the 8- to 15-week age group are susceptible to artificial transmission of gray-patch disease virus by the scratch-inoculation technique. When inoculated with the virus by scratch-inoculation, they do not die, but develop typical gray-patch lesions along the line of scratch. Thus, there appears to be a distinct difference in susceptibility in young green turtles, depending upon their age. Very young, green sea turtles (2-6 weeks of age) appear to be highly susceptible, and die as a result of artificial inoculation, whereas somewhat older turtles (8-15 weeks of age) contract the disease in its epidermal form, and most of the time do not die as a result of the artificial inoculation. The reason for this difference in susceptibility is not known.

Turtles over 1 year of age do not have the natural lesions of gray-patch disease. Interestingly, we have not been able to artificially transmit gray-patch disease to this group of turtles by injection or by scratch-inoculation. This indicates that turtles which have gone through previous epizootics of this disease have become immune.

Origin of Gray-Patch Disease Virus

Another point of consideration, important both for prevention and treatment of this herpesvirus disease, is the question of origin of the virus. Did it arrive with the original eggs from the beaches in the wild, or is it a virus that is maintained on the farm, and transmitted to each successive group of hatchlings?

To answer this, we have taken several groups of eggs, hatched them away from the contaminated environment of the farm, raised the hatchlings in different locations, and monitored them for naturally acquired gray-patch lesions. Different groups of turtles have been raised in Bimini, The Bahamas, in Arizona, and Virginia Key and Pigeon Key, Fla. Each group of turtles raised in these diverse locations, under different environmental conditions, has developed the lesions of gray-patch disease. In general, however, because these animals were not in a stressful environment, the lesions were of the less extensive, pustular form. This appears to indicate that the virus is indeed a natural virus of the green sea turtle, and possibly occurs in the wild. To prove this, however, it would be necessary to raise stocks of turtles from many more parts of the world and determine if they also have the virus.

Effect of Temperature on Gray-Patch Disease

It appears that the total stress load on the animals determines both the extent and outcome of gray-patch disease. As mentioned above gray-patch disease is more severe in summer months when the water temperature of the tanks is 4°-5°C higher than in the cooler, winter months. A series of experiments was carried out to determine the exact effect of water temperature upon the onset and severity of natural gray-patch disease in young turtles. We hatched a large number of turtles away from the farm and performed a heat induction experiment in an artificial tank environment.

The experiment was carried out in Tucson, Ariz., under carefully controlled, experimental conditions. Four groups of 8-week-old green sea turtles, randomly selected, were subjected to a series of temperature changes and monitored for subsequent gray-patch dis-

ease development (Haines and Kleese, 1977).

One group of 52 animals was held in water at a constant temperature of 25°±0.5°C. These were the control animals. A second group of animals was subjected to a water temperature increase from 25° to 30°C at a rate of 1° per day. They were then left for 3 days at 30°C and subjected to a decrease from 30° to 25°C at the same rate of 1° per day. A third group was subjected to the same gradual temperature increase of 1° per day from 25° to 30°C but maintained at 30°C for the duration of the experiment. A fourth group was taken immediately from water at 25°C and placed in water at 30°C, left for 4 days at 30°C, and then returned directly into water at 25°C. These animals were, in essence, temperature "shocked." Each group was observed for the development of gray-patch lesions over a period of 48 days.

The animals subjected to temperature shock treatment and those which were subjected to a gradual increase to 30°C, and maintained at 30°C, had an earlier onset of disease, with more severe lesions, than either the control group or the group of animals which were subjected to a gradual increase to 30°C and then brought back down to 25°C. This is good evidence that heat is one of the stress factors which induce gray-patch disease and that higher water temperatures tend to increase the severity of the lesions. It must be pointed out that this experiment was carried out under ideal conditions for the turtle, in terms of stress. There are no other stress factors that were applied to the animals other than water tempera-ture changes. Under actual aquaculture conditions on the turtle farm, a variety of other stress factors may have a cumu-lative effect upon the severity of the disease and upon the mortality rate. It appears, however, that water tempera-ture may be the major stress factor that triggers gray-patch disease.

Vaccination Attempts With Gray-Patch Disease Virus

We have also produced an inacti-vated vaccine from gray-patch disease virus (Haines et al. unpubl. manuscr.[2]). Scrapings from animals with active dis-ease were semipurified and the virus was injected into young, susceptible animals which were subsequently chal-lenged by the scratch-inoculation technique. Intramuscular injections of dead gray-patch virus protects against challenge with active gray-patch mate-rial in inoculated animals. These pre-liminary trials have shown that the inac-tivated virus vaccine is feasible for farm-wide use.

Attempts to Isolate Gray-Patch Disease Virus

We have carried out many attempts to isolate the turtle herpesvirus in cell culture. Primary green turtle kidney cells, primary green turtle lung cells, and primary green turtle skin cells have been used as potential susceptible cell types. We have also used a large number of other mammalian, reptilian,

and fish cell lines. The only cells in which we have been able to achieve a constant infection showing consistent cytopathic effect are green turtle skin cells which have been passed in the laboratory numerous times (Koment and Haines, in prep.). Very recently, my associate, Roger Koment, has suc-ceeded in taking material from these turtle skin cultures, and successfully in-fected young turtles by the scratch-inoculation technique. The inoculation produced new gray-patch lesions in the animals. So, it appears that the virus does, in fact, replicate in some cell cul-tures, although the replication is difficult to detect by normal methods.

SUMMARY

In summary, an epizootic herpes-virus disease of farmed green sea turtles has been described. This disease, which has been named gray-patch dis-ease, affects young sea turtles, with the highest mortality and most severe le-sions seen in animals that have been subjected to higher water temperatures. Controlled laboratory experimentation proved that high water temperature af-fects both the onset and severity of the lesions. An inactivated virus vaccine appears to protect against challenge with the herpesvirus, and it is possible that the disease may be controllable by vaccination of young turtles.

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Incidence of Disease in Warmwater Fish Farms in the South-Central United States

F. P. MEYER

ABSTRACT—Case records for the years 1963-68 from the diagnostic laboratory at the Fish Farming Experimental Station, Stuttgart, Ark., were studied to determine seasonal incidences and/or interrelationships between various disease conditions. Seasonal occurrences were recorded for Trichodina, Ichthyophthirius, Scyphidia, Plistophora, Costia, Gyrodactylus, Dactylogyrus, Cleidodiscus, *and* Lernaea. *Summer infections of* Aeromonas hydrophila *showed a relationship to periods of oxygen depletion or low oxygen stresses. Graphs are presented identifying months when disease problems are most likely to occur and methods of avoiding outbreaks are discussed. Relationships between occurrences of particular organisms and water temperature, spawning seasons, and other stress periods are noted.*

The greatest incidence of disease was in April which had almost 50 percent more cases than July, the next highest month. Outbreaks occurred regularly throughout the period from March through July. Seventy-two percent of all cases occurred during these months. Only Lernaea *and myxobacterial infections had their greatest incidence outside this period.*

Warmwater fish culture is, to a major extent, restricted to pond culture. While the incubation of eggs and rearing of larval stages of some species may be done in artificial systems, upwards of 95 percent of all warmwater fish produced in the United States are reared in pond facilities.

INCIDENCE OF DISEASE ON FISH FARMS

The incidence of disease on fish farms has been documented by Meyer (1970) and Rogers et al. (1971). April is the most troublesome month of the year. Nearly one-fifth of all case histories recorded over a 5-year period at the Fish Farming Experimental Station, Stuttgart, Ark., occurred during this period. The number was one-third greater than during July, the next highest month. The season from 1 March through July is a continuous period of

potential danger to fish stocks. This period embraces the spawning seasons of golden shiners (*Notemigonis crysoleucas*) and catfish (*Ictalurus punctatus*) and reflects the problems associated with infections in or on very young fish. Shiners spawn in late March, April, and May. Catfish begin spawning in late May and may continue through June into July.

It is relevant that large numbers of fish are handled during March and April, either for marketing, for stocking of broodfish, or for stocking fingerlings in rearing ponds. This handling stress is believed to be a factor in initiating some of the problems during this time of the year.

The low number of disease cases during December results from several factors. The harvest of fish from rearing ponds drastically reduces the number of stocked ponds during November and

December. Fish which are not marketed at harvest are usually placed in holding ponds for winter storage, often under heavily crowded conditions. Apparently, parasite burdens do not have ample time to develop to troublesome levels in storage ponds until some time in January. The jump from three cases in December to 64 in January is believed to be a direct reflection of winter storage conditions (Meyer, 1970).

INCIDENCE OF PROTOZOAN INFECTIONS

Protozoans constitute the major etiological agents and were involved in 53 percent of the observed cases. Their primary effect is greatest on very young fish; hence, the peak incidence of protozoan epizootics occurs during and immediately following spawning periods of both shiners and catfish (Fig. 1).

Danger of parasitism is high from March through July. Young fish are vulnerable to diseases of parasitic origin and are involved in the majority of cases. Since the danger period embraces the spawning period of both shiners and catfish, the importance of having parasite-free broodfish cannot be overemphasized. Treatments should be made periodically throughout the year to keep parasite burdens on broodfish to a minimum. The effectiveness of prophylactic treatments is emphasized by a lower incidence of parasitic diseases on fish farms where prophylactic measures and sanitation were practiced.

INCIDENCE OF BACTERIAL INFECTIONS AND OXYGEN DEPLETIONS

Parasitic diseases cause the greatest number of cases but their overall economic effect is much less than that of bacterial disease (Fig. 2). Problems of parasitic etiology are usually less acute (although nonetheless serious) and the owner often has an extended

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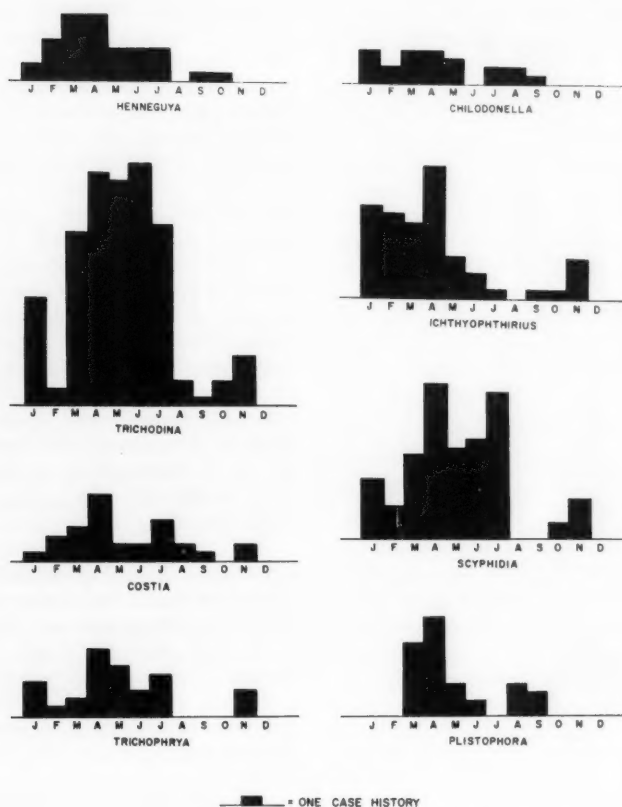
Figure 1.—The monthly incidence of selected protozoan infections reported on warmwater fish farms during a 5-year period.

period during which diagnosis and treatment are possible. This is not true in the case of bacterial disease. The course of most bacterial diseases is rapid and terminal unless early diagnosis and prompt treatment are applied (Robinson et al., 1970).

Next to the danger of oxygen depletion, bacterial disease must be considered as a major threat to the successful production of edible-sized fish.

Pseudomonas infections occur infrequently throughout the year but are not abundant enough to indicate any periodicity.

Aeromonas hydrophila infections are most prevalent during June, July, and August, but a peak has also been noted in April. This two-peak occurrence

reflects differences in hosts. Outbreaks in April are usually associated with spawning activity of golden shiners but may also reflect temperature stresses as suggested by Snieszko (1954). Epizootics in June, July, and August involve catfish in rearing ponds and coincide with periods when oxygen levels are lowest. The bacterial infections occur from 10 days to 2 weeks following the period when the fish have been subjected to stresses associated with low levels of dissolved oxygen. While total depletions cause mortalities due to suffocation, oxygen levels of 3 ppm or less will cause severe stress.

The apparent coincidence of parasitic reproduction with fish spawning indicates that fish culturists should

examine stocks of fry and small fingerlings for the presence of parasites. Failure to do so can result in major losses.

Parasite problems encountered in young-of-the-year fish can, with few exceptions, be traced to parasitized broodfish. Some farmers, who make no effort to reduce the number of parasites on adult fish prior to spawning, suffer losses of 1-hour-old-catfish fry due to *Trichodina*. Open-pond spawning methods in which adults and their offspring are left in the same pond throughout the growing season also result in more parasite problems than the practice of moving egg-laden mats (in the case of shiners) to rearing ponds or the use of artificial hatching units for catfish.

Trichodina is the most commonly encountered protozoan parasite and is especially serious to young fish. Newly hatched channel catfish fry have been observed to be so heavily infected with this parasite that the fish do not survive beyond 1 hour. Young golden shiners which are heavily infected usually fail to feed. *Trichodina* also causes problems to crowded fish held in winter storage ponds, as evidenced by the number of cases during January.

Farmers who hold fingerlings and broodfish in the same ponds often encounter "Ich" disease. The occurrence of *Ichthyophthirius*. Farmers who are successful in preventing the entry of wild fish have fewer parasite problems.

This low oxygen stress is considered to be a major factor in outbreaks which occur in rearing ponds and gives a further indication of the relationship of disease to water conditions in the ponds. Low oxygen levels in ponds pass unobserved on many fish farms and the role of low oxygen in initiating bacterial infections is often unsuspected. Periods during which low oxygen levels are common correspond to the periods indicated on the graph for oxygen depletions. The number of occurrences of sublethal oxygen depletions, however, is actually much higher.
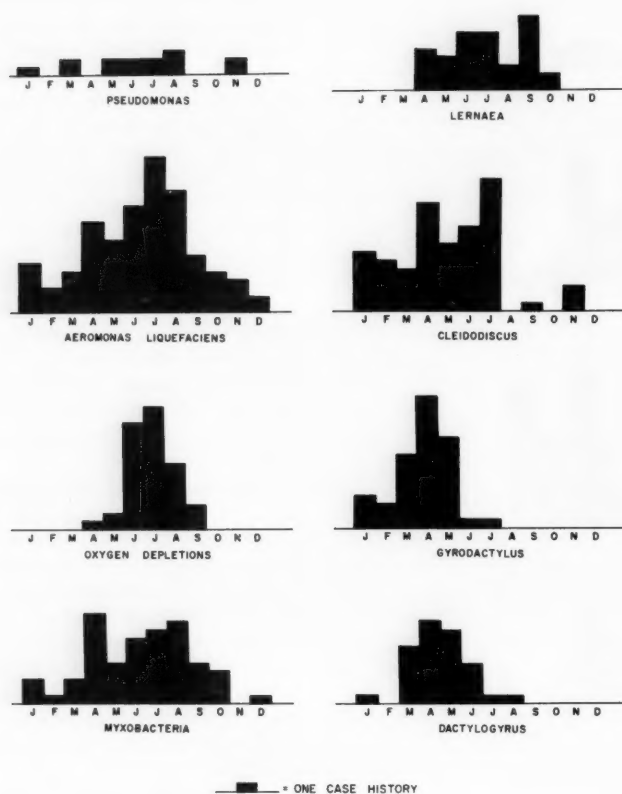
Figure 2.—The monthly incidence of bacterial infections, oxygen depletions, *Lernaea* and monogenetic trematode infestations reported on warmwater fish farms during a 5-year period.

Myxobacterial infections are common on golden shiners during their spawning season and occur on channel catfish fingerlings after handling in April. A second and somewhat broader peak involves both species of fish during June, July, and August, again corresponding closely to periods when pond conditions are poorest and oxygen levels are lowest. Although ice cover is uncommon in the fish farming area, outbreaks of myxobacterial infections occur whenever ice persists for longer than 7 days on storage ponds. Deteriorating pond conditions under the ice are believed to be related to the onset of infections due to myxobacteria.

ANTIBIOTICS USED AS PROPHYLACTICS

Infections associated with stocking or handling can be reduced if handling is delayed until the fish have received a regimen of antibiotic treatment. Although spring stocking may be delayed slightly, the increased survival rate should more than justify the delay. Stresses due to handling may cause a bacterial infection to progress from a chronic to an acute stage. Similarly, fish handled in the summer frequently develop infections similar to bovine shipping fever syndrome. Antibiotic medication for 10 days prior to handling serves as an excellent preventive measure.

Stresses associated with low oxygen levels can be combated in a similar way. On well-managed fish farms, dissolved oxygen levels are usually monitored regularly. Anytime the level has dropped rapidly, the farmer should realize that the fish have been subjected to a significant stress which will likely lead to bacterial disease. Again, antibiotic therapy is indicated.

Losses of broodfish can usually be traced to physiological stresses and physical abuse during the spawning season. Feeding adult fish a medicated ration for a 10-day period prior to handling or to the onset of spawning should reduce losses significantly. If this is not possible, an intraperitoneal injection of 25 mg of Terramycin[1] per pound of body weight will also combat *Aeromonas* infection.

While the role of oxygen levels in the incidence of *A. hydrophila* infections appears obvious, the carbon dioxide role of spawning stresses, temperatures, pH changes, high carbon dioxide, sulfides, and other decomposition products associated with low oxygen levels should also be considered as possible factors.

TREATMENT DELIVERY SYSTEMS

Three basic treatment regimens are currently used in warmwater fish culture.

Bath Treatment

A bath treatment with selected chemicals for the removal of external parasites is the most common treatment regimen. These baths may be: 1) at low treatment rates for extended or indefinite periods; or 2) at high rates for rigidly specified times. Such applications are inefficient and expensive. Technique 1) is usually used for pond applications. A ton of catfish in a 1-acre pond averaging 4 feet deep would constitute only 0.018 percent of the mass

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[1]Mention of trade names or commercial products does not imply endorsement by the National Marine Fisheries Service, NOAA.

by weight. This mean that 99.98 percent of the chemical is not utilized. The cost factor is readily apparent. In technique 2), the use pattern is restricted to small tanks or raceways. While chemical costs are usually low, labor and time requirements are significant.

Oral Treatment

Internal diseases of fish can be treated only if a delivery system is available to introduce the drugs internally. This means that either the fish must accept the medication voluntarily (as with their feed) or that the medication must be provided manually (usually by injection). The former technique can be used on those species which accept artificial feed only so long as the fish are not too sick to eat. Success with oral delivery systems is directly related to early, accurate diagnosis of the problem. Cost of oral delivery systems is low and effective. Labor requirements are also low.

Injection Treatment

Treatment by injection is currently practiced in the United States only on broodstock. The high value of each individual animal more than offsets the labor and time requirements of this delivery system. The technique is used widely in Europe as a measure to provide protection to young carp from bacterial disease during handling and stocking. It has proven effective and economically feasible in Europe because of low labor costs. Injection techniques require the handling of each fish. Even with the most modern injection systems available today, time and manpower requirements preclude the use of injection delivery systems on other than broodfish in the United States.

Further Delivery Systems Development Needed

A review of these treatment systems indicates the following unfilled needs.

1) No delivery system exists for treating systemic bacterial infections in pond-spawned fishes that do not accept artificial feeds.

2) No economical delivery system exists for mass injection of large numbers of fish prior to stocking.

3) No efficient treatment system exists for treating fish in ponds for external parasites or bacterial infections.

Considerations should also include delivery of potential vaccines for the immunization of fish. Although vaccines are still in formative stages, it is relevant that delivery systems be developed. Under the existing state of knowledge, it appears that the delivery of antigenic materials faces the same limitations as chemotherapy. New delivery systems and alternate cultural practices are needed if significant gains are to be achieved in improving fish health.

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Enteric Red Mouth Disease
(Hagerman Strain)

R. A. BUSCH

ABSTRACT — Enteric red mouth (ERM) disease of salmonid fishes is reviewed in terms of description of the etiological agent, pathogenesis and diagnosis of the clinical condition, and epizootiological considerations pertinent to its effective control. Recent studies defining the asymptomatic carrier state of the disease are discussed. Enteric red mouth disease is shown to establish an asymptomatic carrier state infection in the lumen of the lower intestine in 50 to 75 percent of epizootic survivors 60 to 65 days postinfection. This chronic carrier state is maintained for more than 102 days in the experimental population. A cyclical intestinal shedding pattern develops with a periodicity of 36 to 40 days and serves to cause regular reinfection and mortality.

A serological presumptive screening test for ERM is described. The system is shown to be sensitive and specific and readily adapted to the rapid screening of large numbers of individual small volume sera for presumptive evidence of specific disease association at a minimum cost and without sacrifice of fish over 15 cm in length. Based upon serological screen field data, ERM is shown to consistently develop a 2.0 to 2.7 percent carrier incidence among populations following epizootic infection. The humoral immune response of rainbow trout to various antigenic preparations of the ERM bacterium is examined. Various soluble protein fractions are shown to be more immunogenic than lipopolysaccharide fractions. A particulate cell wall antigen and several other killed whole cell antigens resulted in high titers while a heat-killed whole-cell preparation is shown to be nonimmunogenic. Serum agglutinin titers declined at a monthly rate of 9.8 percent following induction and were detectable for more than 9 months. Evidence is given to support the theory that serum agglutinins in trout are IgM-like macroglobulins.

Enteric red mouth (ERM) disease was first recognized as a cause of mortalities in rainbow trout, *Salmo gairdneri*, in the late 1950's in the Hagerman Valley of Idaho. Subsequently, the disease was studied by Ross et al. (1966) and Rucker (1966). These workers were unable to give the etiological agent a more definitive taxonomic position, but based upon morphological, biochemical, and serological data, they placed it in the family Enterobacteriaceae.

EPIDEMIOLOGY

The ERM bacterium was originally referred to as the "RM bacterium." This terminology tended to confuse it with other bacterial pathogens that

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cause a similar clinical syndrome. McDaniel (1971) proposed the name Hagerman red mouth or HRM, describing its original range. In 1975 the Fish Health Section of the American Fisheries Society chose to remove this onus on the Hagerman Valley and called it enteric red mouth disease, indicating that the causative agent is one of the two major enteric pathogens of fish in fresh water: the other being *Edwardsiella tarda*. Enteric red mouth disease has also been referred to in the literature as red mouth disease, red vent disease, and bacterial hemorrhagic septicemia, but in many instances, authors were describing a clinical syndrome that was often of diverse or multiple etiology rather than the etiological agent itself.

In 1966, ERM was found to be endemic to the Hagerman Valley of Idaho as well as the States of California, Nevada, Arizona, and Colorado. When one studies the epidemiology of this geographically isolated disease and its dissemination, one often finds that it was introduced with subclinically infected stocks of fish acquired from an area where it is endemic. As an example, in the middle and late 1960's, the trout industry in the Hagerman Valley shipped large numbers of asymptomatically infected live fish to various western states for grow-out and fish-out operations. During that time the disease was widely disseminated, and in recent years economically important geographical range extensions and epizootics of ERM were documented to occur due to transporation of asymptomatically infected stocks into previously disease-free areas (Wobeser, 1973). By 1970, the originally recognized range of occurrence had expanded to include Alaska, Oregon, Utah, Washington, and Wyoming. More recently, it has been reported from British Columbia, Montana, Nebraska, Ohio, Saskatchewan, and Tennessee. The disease has since been reported from various other places outside of North America, particularly Italy, but these reports, to my knowledge, have not been serologically confirmed. It is significant to note that ERM still remains comparatively limited in its geographical distribution on

a worldwide watershed basis and, therefore, demonstrates an excellent potential for preventative management and an eradication type of program in comparison to other more ubiquitous fish pathogens.

ECONOMIC EFFECTS

Some causative agents of fish diseases are ubiquitous in their occurence and even free living in certain instances, while other pathogens are fairly fastidious and geographically isolated, as is the case with ERM. This will have a definite practical bearing on how we are going to prevent, manage, treat, and handle stocks of fish infected with ERM and reduce the adverse economic impacts of the disease on the trout industry.

In the Hagerman Valley, commercial fish farms located along a 35-km stretch of the Snake River canyon produce about 80 percent of the commercial rainbow trout in the United States. These are private and highly competitive operations. It is difficult to get accurate figures, but this production amounts to over 9,000 metric tons (t) of rainbow trout a year. One hatchery that has just been put into operation has an annual production capacity of 2,250 t of trout based upon a flow of 9.20 m³ per second of spring water from the aquifer of the Snake River plain.

Araji (1972) did an economic study on the impact of disease on the trout industry in the Hagerman Valley and estimated an annual loss of approximately $750,000 or roughly 10 percent of the total production costs. I have seen loss estimates as high as 35 percent of production costs and up to $2½ million annually depending upon the operation and management involved. It is interesting to note a recent study by Klontz and King (1975) on the exact nature of these losses. If you were to follow losses due to disease in egg, sac fry, swim-up and fingerling stages, and on up to marketable 15- to 30-cm fish, the total number of mortalities are greatest in the egg through swim-up fry stages and decrease as the fish get older. However, while the mortalities in marketable size fish represents only 14 per-

cent of the total mortalities, it amounts to 76 percent of the costs in dollars. The mortality in the small fish in these hatcheries is mainly attributed to bacterial gill disease and other management-related conditions as well as infectious pancreatic necrosis, a presently untreatable viral disease. In comparison, losses in the marketable size fish are attributed to ERM and other treatable bacterial diseases. This concept is of primary importance in the consideration of the economics of disease prevention and treatment in these stocks.

CAUSATIVE ORGANISM

Morphologically, the ERM bacterium is a 1.0×2.0 to 3.0 μm, gram negative, monotrichously flagellated rod which forms a smooth, circular, raised, entire, nonflourescent, nonpigmented colony on nutrient agar with a buterous type of growth. It is non-aerogenic, fermentative, cytochrome oxidase negative, catalase positive, and typical of enteric bacteria in general and is indole negative, methyl red positive, Voges-Proskauer negative, and citrate positive. This brings it rather closely down to the Erwinia group of the family Enterobacteriaceae. Serologically, antigenic similarities have been found with the somatic O antigens of atypical Erwinia groups 26 and 29 (Ross et al., 1966; Cisar, 1972).

MORTALITY AND TRANSMISSION

Enteric red mouth disease has been known to cause mortality in all trout and salmon. The most susceptible host seems to be rainbow trout, with mortality commonly running from 25 to 75 percent during the course of an untreated epizootic. Populations of brook, *Salvelinus fontinalis*, and brown trout, *Salmo trutta*, seem to be more resistant, with 5 to 10 percent mortality common. The bacterium has not been isolated from warm-water fish, even under experimental laboratory induction. The disease is commonly endemic and survives for long periods of time in organically rich waters and, consequently, lends itself quite well to enzootic infec-

tion under intensive culture situations. It is also commonly found as a clinically asymptomatic carrier-state infection.

Transmission of ERM occurs through the water from feces of infected fish. Pathogenesis of infection varies from peracute to chronic depending upon the temperature, stress, species, age, and so forth. Peracute to acute infection usually occur in the spring and early summer, usually in young-of-the-year fish, during periods of rising water temperatures, and increased handling stress. Mortalities usually commence 4 to 8 days following exposure and run between 50 and 70 percent during a 30- to 60-day course of clinical infection. Acute to subacute infections usually occur in yearling fish in the fall and early winter with declining water temperatures. Mortality usually runs about 10 to 50 percent in a 2- to 6-month period. Chronic infection results in very low levels of mortality, approximately 10 percent. However, these losses are often incurred in very valuable market-sized or mature brood stock fishes and, economically, a 10 percent mortality can be significant.

CLINICAL SIGNS AND PATHOLOGY

The clinical pathology of ERM is quite characteristic of bacterial hemorrhagic septicemias in general. By definition, ERM could best be referred to as a bacterial hemorrhagic septicemia of salmonid fishes, caused by the ERM bacterium, having a very well defined geographical range of distribution and endemic occurrence, and capable of inflicting severe mortality.

The common external clinical manifestations include subcutaneous hemorrhaging along the base of the fins and in or about the oral cavity and anus. Exophthalmos is brought about by tissue edema. This condition is noted to often start unilaterally and develop to a bilateral involvement (Fig. 1). Histopathological examination demonstrates an edematous type of lesion of the choroid gland of the eye with an intraocular accumulation of fluid. This edema and increased intraocular fluid

pressure induces the exophthalmic condition and eventually results in rupture of the eye with ensuing lens opacity and blindness (Fig. 2). Infected fish will physiologically darken in color and are easily identified in populations suspected as infected, particularly as carriers.

Internal gross pathology is distinguished by petechial hemorrhage of the visceral organs and tissues including the liver, pancreas, adipose tissues, swim bladder, various coelomic mesenteries, and body musculature. Gross tissue edema is noted in the kidney, liver, and spleen along with hemorrhagic reddening of gonadal tissues and the distal ends of the pyloric caeca (Figs. 3-6). The gross pathology of the lower intestine is probably the most significant clinical diagnostic sign of ERM. The intestine becomes inflamed, flaccid, translucent, hemorrhaged, and distended with a serosanguineous yellow mucoid material consisting of necrotized intestinal mucosa heavily loaded with the pathogen.

The histopathology is typical of a hemorrhagic septicemic type of infection and, in the acute form, the bacterium is commonly found in the peripheral blood (Fig. 7). The hematological picture is characterized as an acute macrocytic, hypochromic anemia with leukopenia, resulting from necrotic destruction of hematopoietic and reticuloendothelial tissues, and an increased clotting time as well as a plasma aproteinemia attributed to glomerular nephritis and necrosis of the intestinal mucosa. This condition results in a decrease of the plasma colloid osmotic pressure producing gross systemic tissue edema as well as disruption of the ionic balance.

The acute form of the disease is distinguished by loss of capillary structural integrity, tissue edema, and loss of osmotic and ionic homeostasis. This condition results in a rapid course of infection of 4-10 days with minimum gross clinical pathology. The chronic form is characterized by localized tissue hemorrhage and necrosis, a decline in nutritional condition, and secondary infection. Petechiation results from a loss of capillary integrity and erythro-

Figure 1.—Bilateral exophthalmos in a rainbow trout, *Salmo gairdneri*, due to gross tissue edema caused by acute septicemic infection with enteric red mouth disease.

Figure 2.—Rupture of the eye, lens opacity, and blindness in a rainbow trout, *Salmo gairdneri*, due to increased intraocular fluid pressure and edema caused by acute septicemic infection with enteric red mouth disease.
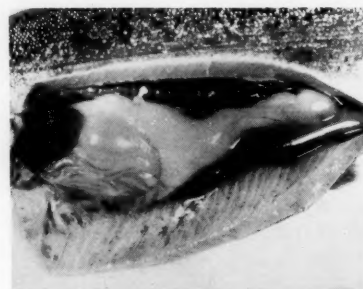
Figure 3.—Generalized petechiation of visceral organs and tissues of a rainbow trout, *Salmo gairdneri*, due to acute septicemic infection with enteric red mouth disease.

Figure 4.—Petechial hemorrhage in the liver of a rainbow trout, *Salmo gairdneri*, due to acute septicemic infection with enteric red mouth disease.
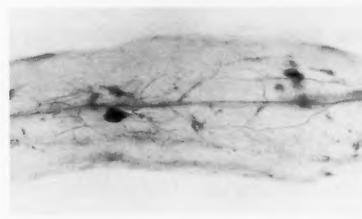
Figure 5.—Hemorrhagic plaques in the swim bladder of a rainbow trout, *Salmo gairdneri*, due to an acute septicemic infection with enteric red mouth disease.

Figure 6.—Petechial hemorrhage of the lateral musculature and visceral mesentery of a rainbow trout, *Salmo gairdneri*, due to an acute septicemic infection with enteric red mouth disease.

cytic congestion of capillary beds and blood sinusoids (Fig. 8). The intestinal tract demonstrates a progressive necrosis and sloughing of the mucosa proceeding down to the stratum compac-

tum (Fig. 9). This situation is highly conducive to secondary infection and complication of the clinical picture by multiple etiologies.
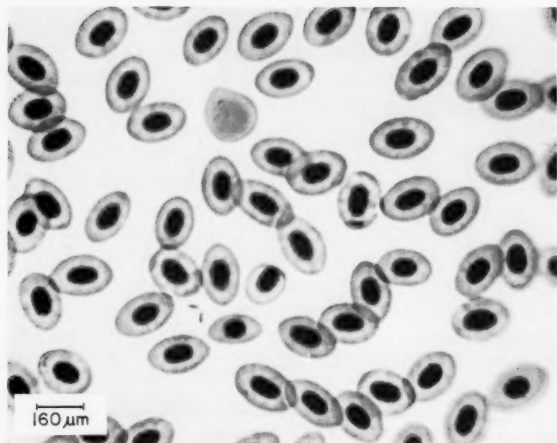
Figure 7.—Peripheral blood smear of rainbow trout, *Salmo gairdneri*, demonstrating the presence of the enteric red mouth disease bacterium as the causative agent of an acute bacteremic infection. (Wright-Giemsa stain.)
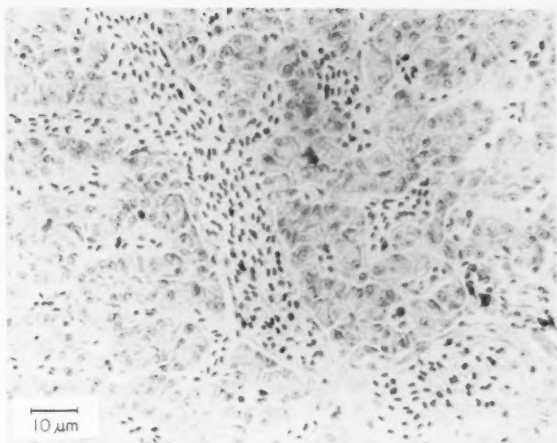
Figure 8.—Erythrocytic congestion of capillary beds and blood sinusoids and tissue edema of the liver of a rainbow trout, *Salmo gairdneri*, due to acute septicemic infection with enteric red mouth disease. (Hematoxylin and Eosin stain.)
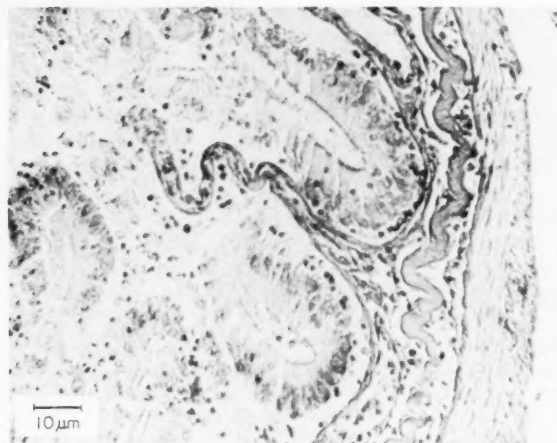
Figure 9.—Mucosal necrosis and sloughing in the pyloric caeca of a rainbow trout, *Salmo gairdneri*, due to acute septicemic infection with enteric red mouth disease. (Hematoxylin and Eosin stain.)

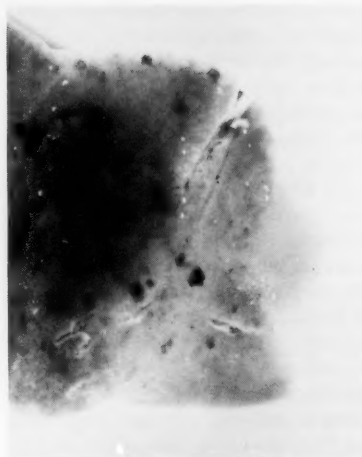Figure 10.—Mortality, gross pathology, and recovery of the enteric red mouth bacterium in correlation with the quanitative serum macroglobulin response during periodic sampling following intraperitoneal infection of rainbow trout, *Salmo gairdneri*.

ASYMPTOMATIC DISEASE CARRIER STATE

The establishment of asymptomatic disease carrier state infections is important to the maintenance of fastidious pathogens within a population in terms of functioning as a constant reservoir for recurrent infection. With the advent of modern husbandry practices to the field of fish culture, the widespread transportation of fish stocks has resulted in the transplantation of economically important fish pathogens to previously uninfected watersheds. These consequences are recognized as being increasingly important to the prevention, control, management, and treatment of viral and bacterial fish diseases. However, management practices depend upon the poorly defined or understood clinical picture of the carrier condition. More importantly, proposed programs and procedures of fish disease inspection, certification, and control are based

Figure 11.—Mortality, gross pathology, and recovery of the enteric red mouth bacterium in correlation with the quantitative serum macroglobulin response during periodic sampling following an immersion mode of infection of rainbow trout, *Salmo gairdneri*.

Figure 12.—Relative rates of recovery of the enteric red mouth bacterium from selected indicator organs during periodic sampling following intraperitoneal infection of rainbow trout, *Salmo gairdneri*.

Figure 13.—Relative rates of recovery of the enteric red mouth bacterium from selected indicator organs during periodic sampling following an immersion mode of infection of rainbow trout, *Salmo gairdneri*.

upon a statistically significant sampling size to determine the suspected carrier incidence. Presently, the carrier incidence is intuitively estimated for most diseases.

In recent studies conducted at the University of Idaho and supported by the Idaho Agriculture Experiment Station, I was able to further elucidate the asymptomatic carrier state of the ERM disease. Using yearling rainbow trout with no known history of ERM, I was able to clinically follow the pathogenesis of infection through development of the asymptomatic carrier condition and study the dynamics of its establishment, mechanisms of maintenance, and levels of incidence pertinent to significant sample size determination, surveillance, and diagnosis. Experiments were run at 14.5°C which is the year-round water temperature of the fish farms of the Hagerman Valley. I used two modes of infection: A parenteral injection with an 18 hour culture of the virulent organism at an

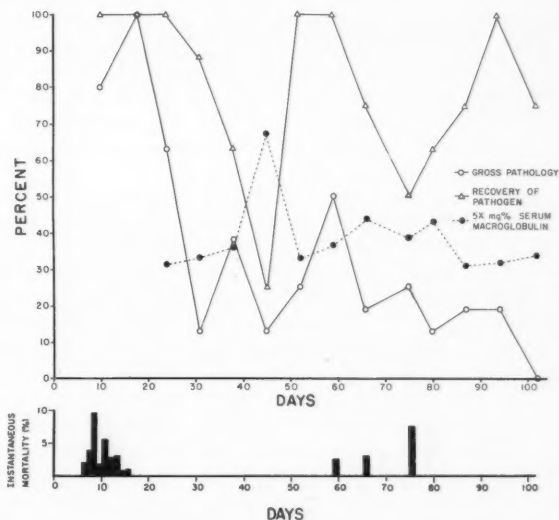LD_{30} dose of 18 organisms per fish, and fish immersed for 1 hour in a bath suspension of 10^6 ERM organisms per milliliter at an elevated temperature of 20°C. Pathogenesis was followed by collection of mortalities, weekly subsampling of survivors, determination of gross clinical signs, serological and electrophoretic analysis of serum protein macroglobulins, and pathogen isolation from the anterior kidney, liver, spleen, and lower intestine.

In the parenterally infected population, a 27 percent total mortality was induced during the 102-day course of observation. Mortalities typically commenced on day 6 postinfection, reached a maximum on day 9, and ended on day 15 with recurrent mortalities between 59 and 75 days postinfection (Fig. 10). The immersion mode of infection induced only a 2 percent total mortality after 7 days (Fig. 11). However, both modes of experimental exposure induced 100 percent infection of the experimental population at 21
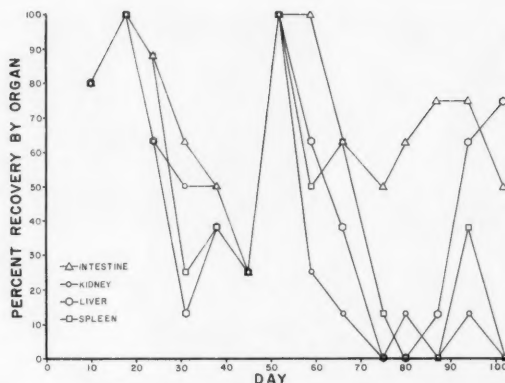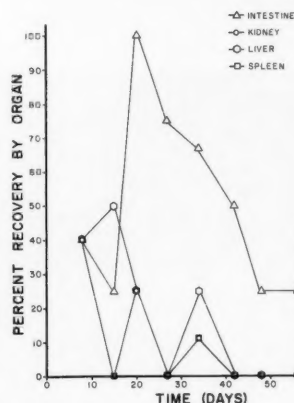
days as demonstrated by the relative tissue recovery rates of the pathogen (Figs. 10, 11).

During the first 30 to 60 days postinfection, the bacterium was routinely recovered from all indicator tissues sampled from both populations (Figs. 12, 13). The ERM bacterium was shown in Figures 12 and 13 to readily establish an asymptomatic carrier state infection, by localizing in the lumen of the lower intestine in 50 to 75 percent of the survivors, 60 to 65 days postinfection, and

remaining there for more than 102 days. The relative intestinal recovery rate and presence of the pathogen within the infected populations was also shown in Figures 12 and 13 to be of a cyclical nature, with a periodicity of 36 to 40 days. Only 25 to 50 percent of the asymptomatic carrier infections established would be clinically identified for inspection and certification purposes by using classical attempts at isolation from the kidney and spleen during the low points of the cycle.

Cyclical Nature of Carrier Infections

On the basis of pathogen recovery, gross pathological changes, and mortality rates, it appears that a regular 36- to 40-day cycle of intestinal shedding of the ERM bacterium occurs and precedes the recurrence of systemic involvment and mortality by 3 to 5 days. This type of cyclical shedding could precipitate continuous recurrent mortality in a naturally infected susceptible population throughout the year. The actual periodicity and mortality levels of these shedding cycles would be altered by seasonal variations in water temperatures, loading factors, handling, and other stresses as well as natural resistance and immunity of the population. This hypothesis is substantiated by the observations of McDaniel (1971), who demonstrated cyclical mortality patterns of similar periodicity to occur throughout the year in a large, untreated hatchery population chronically infected with ERM disease (Fig. 14).
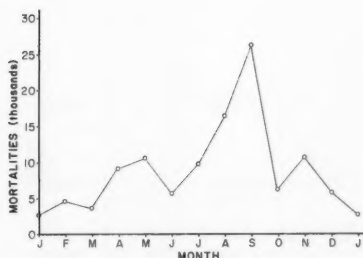
Figure 14.—Monthly mortality rates attributed to endemic enteric red mouth disease infection of an untreated hatchery population of rainbow trout, *Salmo gairdneri*. (from McDaniel, 1971).

Many demonstrated aspects of the clinical course of induced ERM infection have the classical appearance of an acquired protective immune response. In particular, the decrease in mortality and relative rates of gross pathological changes occurred 14 to 21 days postinfection. This time period corresponds to the expected lag phase and logarithmic induction period of a protective immune response at 14.5°C. Electrophoregram analysis demonstrated a quantitative serum macroglobulin response as shown in Figures 10 and 11. However, this quantitative change in serum macroglobulin could not be detected as agglutinating or precipitating humoral antibodies, indicating the possible involvement of a protective monovalent or incomplete immune globulin fraction, possibly functioning in an opsonizing or cytophilic capacity. An induced cellular immunity is most probably responsible for the rapid clearance of the pathogen from the reticuloendothelial tissues and its localization in the lumen of the lower intestine where it is comparatively removed from macrophage and immunoglobulin activity.

This insight into the establishment of a clinically asymptomatic carrier infection and its demonstrated cyclical nature of shedding could find direct application to management practices of the disease in terms of the timing of handling stress, loading factors, and other "controllable stress." The use of antibiotic or chemotherapeutic agents with a high retention time in the lumen of the gut is indicated rather than other more rapidly absorbed agents for treatment of the carrier condition. Detecting the condition by classical isolation and identification methods (particularly if the lower intestine is not sampled) is difficult, and this may seriously affect surveillance and certification operations. The cyclical nature of the carrier infection also indicates the need for a paired sampling at a 15-day interval to achieve maximum diagnostic efficiency.

Identifying Carriers

Of major consequence to current programs of inspection, certification,

and control is the ability to efficiently diagnose clinical carrier infections. Present methods of classical isolation and identification of the pathogen are wholly inadequate in terms of sensitivity, logistics, time frame, and economics. Programs of this type often involve large numbers of fish which must be certified free of particular disease agents but are often highly valuable and cannot be sacrificed as in the case of brood stocks.

Development of the Passive Agglutination Test

As a result of my experience with the U.S. Public Health Service Center for Disease Control's sylvatic plague surveillance program, I have developed a highly sensitive and specific means of serological disease surveillance requiring a minimum of lethal sampling. The procedure is based upon a passive or indirect agglutination test using microtiter techniques.

The antigenicity of the ERM organism was examined in detail. A pH 6.4 boiled aqueous extraction of a washed 18-hour ERM culture was found to be the most antigenically complete soluble fraction of the organism. Many different substrate particle systems, including latex, bentonite, charcoal, fresh erythrocytes, and fixed erythrocytes were incorporated into the test. Maximum sensitivity was obtained with fresh citrated sheep erythrocytes which were tanned and sensitized with 10 mg% antigen protein at pH 6.4. Test sera were diluted in normal physiological saline with normal rabbit serum added at 1:100. Other substrate particle systems were found to be somewhat less sensitive but could be lyophilized and stored for long periods of time.

Figure 15 demonstrates the agglutination patterns achieved. Note the very sharp end points and negative buttons. Titers are easy to pick out, and the test is well adapted to either screening or titering test sera. In addition to the passive agglutination test, a paired inhibition dilution series utilizing 20 mg% antigen protein added to the serum diluent was run. The inclusion of the
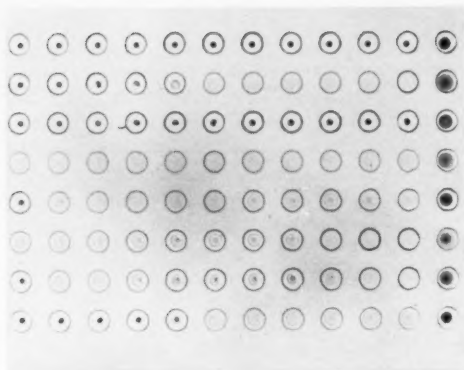
Figure 15.—Typical results of the micromodified passive agglutination test demonstrating diffuse patterns of positive agglutination and negative buttons as well as end point titers of sera dilution series.

Figure 16.—Basic materials necessary for the performance of the micromodified passive agglutination test applicable for screening of large numbers of individual small volume sera for the presumptive serological evidence of specific disease association.

paired inhibition test gave complete control over any nonspecific agglutinating activity for each sample tested.

This type of test lends itself to the rapid field screening of large numbers of individual small volume sera for presumptive evidence of specific disease association at a minimum cost and without sacrifice of fish over 15 cm. Utilizing an unsophisticated hand-operated system costing $100, a reasonably good technician can screen about 500 fish in an 8-hour day against a variety of diseases (Fig. 16). The test can also be readily automated for even greater efficiency. Results can be read immediately following centrifugation or after 2-4 hours of incubation.

Field Application of Passive Agglutination Test

In the fall of 1972, the passive agglutination procedure was field tested in the Hagerman Valley. The fish stocks of eight stations having a history of ERM, but no recent epizootics, and a single station undergoing an active epizootic were examined. Station stocks were first sampled in September, just at the end of the usual seasonal peak in mortality attributed to ERM disease, and then reexamined 60 days later in November.

Initial serological screening resulted in a 38.2 percent incidence of positive

titers for the infected station stocks and a 7.7 percent incidence for all other stations combined. Positive serum titers during this period ranged from 1:16 to 1:32,768. The actual individual titer distributions are summarized in Figure 17 for the two populations. A definite natural split in high and low level titers occurred. High level natural titers of 1:512 or greater (shaded areas of Fig. 17) constituted 19.4 percent of all positive titer (shaded and unshaded combined) from the clinically infected station and 30.8 percent of all positive titers from all other nonclinically infected stations combined during the first sampling date.

As summarized in Figure 18, an 8.4 percent incidence of positive titers for the infected station and an 8.8 percent incidence of positive titers for the other stations were found at the second sampling date. When the natural break between the high and low level titers was examined for the second set of data, high titered sera were absent from the infected station and constituted 21.3 percent of the positive sera from the other stations combined.

It was postulated that the natural break between high titered and low titered sera is indicative of the difference between subacute to chronic clinical infections and the presence of an established carrier population, respectively.

Figure 17.—Natural distribution of serum passive agglutinin titers from hatchery populations of rainbow trout, *Salmo gairdneri*, surviving a recent clinical epizootic of enteric red mouth (ERM) disease (left) and those not clinically infected but grown in an endemic area of ERM infection (right), as sampled in September 1972.
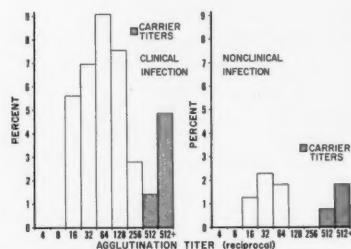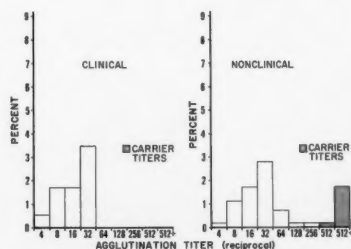

Figure 18.—Natural distribution of serum passive agglutination titers from hatchery populations of rainbow trout, *Salmo gairdneri,* surviving a recent clinical epizootic of enteric red mouth (ERM) disease (left) and those not clinically infected but grown in an endemic area of ERM infection (right), as sampled in October 1972.

This hypothesis is based upon the premise that continued presence of the pathogen over a long period of time, as occurs in an established carrier infection, is necessary to induce high levels of serum agglutinins. The lower titered grouping would then include those survivors which still may harbor the pathogen but have not yet established classical carrier states of infection. Such a hypothesis is supported by the fact that the relative rates of occurrence of the high titered grouping remained constant in the combined stations sampled 60 days apart as shown in the shaded areas of Figures 17 and 18. The 2.7 percent and 2.0 percent respective proportions of the populations seem to demonstrate a constant level of carrier incidence which is consistent with levels of carrier incidence reported for other related diseases.

The overall results from the infected station, as shown in Figures 17 and 18, indicate an initially high rate of infection with a proportionally large number, 6.3 percent, of high titered presumptive carrier fish being present. The second sampling indicated a 31 percent decrease in positive screen titers and the complete lack of any high titered group of sera. This situation could be indicative of a resistant recovering population in which true carrier states had yet to be established. This data could also be attributed to the fact that the second sampling was essentially a different lot of fish from those originally sampled, even though the station was the same.

INDUCED HUMORAL IMMUNE RESPONSE IN RAINBOW TROUT

Induction of a specific immune response in rainbow trout to immunogenic preparations of the ERM bacterium was examined in terms of its nature and dynamics.

Figures 19 and 20 demonstrate the dynamics of a humoral agglutinin response in rainbow trout to extended parenteral exposure to soluble antigenic preparations of the ERM bacterium. Protein-based water soluble extracts are shown in Figure 19 and carbohydrate-based organic solvent extracts are shown in Figure 20. Various preparations included a boiled aqueous extract (BAE), ammonium sulfate supernate (ASS), ammonium sulfate precipitate (ASP), phenol extract (PE), ether-water extract (EWE), and chloroform-water extract (CWE). Humoral passive agglutinating antibodies against protein-based soluble antigens were first detected 13 days after intiation of the first of two series of weekly injections. Specific passive agglutination titers to organic solvent extracts were initially detected in 28 days. All serum agglutinin titers were shown to rise throughout the initial period of antigenic stimulation, but when injections

Figure 19.—The serum passive agglutinin response of rainbow trout, *Salmo gairdneri*, to the administration of water soluble antigenic preparations of the enteric red mouth disease bacterium. (BAE, boiled aqueous extract; ASS, ammonium sulfate supernate; ASP, ammonium sulfate precipitate.)

Figure 20.—The serum passive agglutinin response of rainbow trout, *Salmo gairdneri*, to the administration of organic solvent soluble antigenic preparations of the enteric red mouth disease bacterium. (PE, phenol extract; EWE, ether-water extract; CWE, chloroform-water extract.)
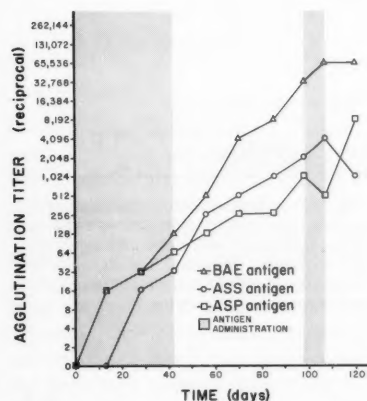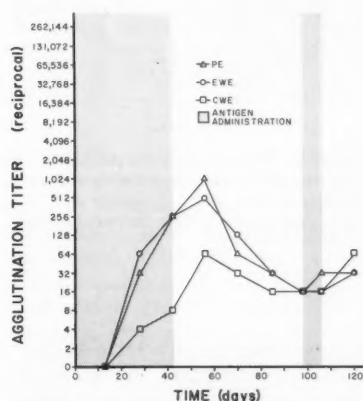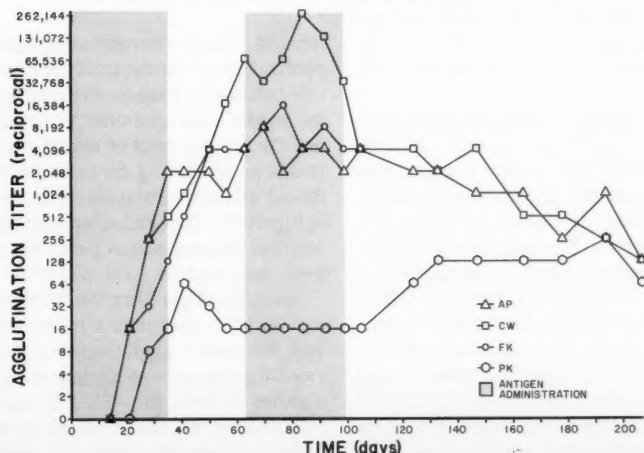
Figure 21.—The passive serum agglutinin response of rainbow trout, *Salmo gairdneri*, to the administration of particulate antigen preparations of the enteric red mouth disease bacterium. (AP, alum precipitate; CW, cell wall; FK, Formalin killed; PK, phenol killed.)

were discontinued, titers to lipopolysaccharide antigens declined while titers to protein antigens continued to rise. Freund's complete adjuvant was used in the first two injections of all preparations.

A second series of weekly injections, commencing on experimental day 98, was shown to have an inductive effect on specific titers of all antigens but not to the extent of a true anamnestic response. Maximum serum passive agglutinin titers of 1:65,531 were obtained from a protein-based antigen (BAE) at 106 days. Comparatively poor responses were obtained from the lipopolysaccharide preparations. The EWE and CWE antigens were extremely toxic to rabbits due to their lipopolysaccharide endotoxin components but were found to have no toxic effect upon trout at the levels administered.

Rainbow trout were also injected with various particulate antigen preparations of ERM including an alum precipitate (AP), washed cell wall suspension (CW), and [1]Formalin-killed (FK) and phenol-killed (PK) whole cells. Humoral passive agglutinins to these particulate antigens were detected at 21 to 28 days into the first series of weekly injections as shown in Figure 21. The CW preparation induced the highest passive agglutinin response with a maximum titer of 1:262,144 in 84 days. The FK and AP antigens gave much the same response. The PK preparation induced a poor serum passive agglutinin response while a heat-killed preparation failed to elicit any type of a passive agglutinin response at all. The high passive agglutination titers induced by various particulate antigens seemed to reach a common maximum titer of 1:4,096 following discontinuation of injections. From this point, the various titers declined similarly at an average rate of 9.8 percent per month and were detectable for more than 9 months.

Quantitative analysis of serial electrophoregrams from rainbow trout
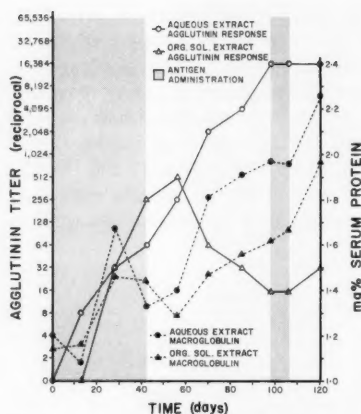
Figure 22.—Passive agglutinin and quantitative macroglobulin response of rainbow trout, *Salmo gairdneri*, serum to aqueous and organic solvent extracts of the enteric red mouth diesease bacterium.

hyperimmunized with soluble antigens of ERM demonstrated an increase in total macroglobulin (fraction F) serum protein during continued antigenic stimulation but no direct correlation could be made to the presence of serum passive agglutinins (Fig.22). However, the macroglobulin response is seen to be directly proportional and responsive to antigenic stimulation and resembles a classical humoral immune response including what appears to be a true anamnestic response at 106 days. This macroglobulin response could be further evidence of a functionally protective monovalent cytophylic or opsinizing globulin not detected by agglutination or precipitation techniques.

Electrophoretic analysis of the time sequence sampling of pooled sera from hyperimmunized rainbow trout produced a normal seven protein component pattern, as shown in Figure 23, consisting of: A prealbumin and albumin complex grouped as peak "A"; a second component, labeled "B," analogous to an alpha-1 peak of lipoprotein; three pseudoglobulin peaks, analogous to alpha-2, beta-1, and beta-2 fractions and called "C," "D," and "E"; and a euglobulin or gamma complex of peaks labeled as the "F"
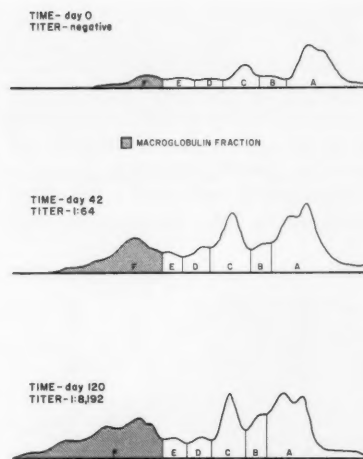
Figure 23.—Sequential electrophoregrams of pooled sera from hyperimmunized rainbow trout, *Salmo gairdneri*, demonstrating serum macroglobulin response. Letters indicate major serum protein peaks based upon relative electrophoretic mobility.
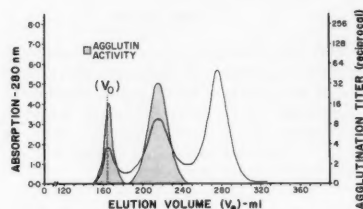
Figure 24.—Gel filtration chromatographic fractionation and passive agglutinin activity determination of hyperimmune mammalian (rabbit) serum proteins.

fraction. Electrophoretic separation of pooled hyperimmune trout sera generally indicated a quantitative increase of the anodic macroglobulin "F" fraction and the development of multiple peaks in this region during the course of continued antigenic stimulation as shown in Figure 23. The gradual appearance of multiple macroglobulin peaks has also been described by Evelyn (1971).

Analysis of gel filtration fractions of anti-ERM hyperimmune rabbit serum with the passive agglutination test indicated the presence of agglutinating antibodies in both the 18S-19S and 7S fractions of the rabbit serum as indicated in Figure 24. However, specific

agglutinin activity was found in only the macroglobulin fraction of the hyperimmune trout serum as shown in Figure 25. No shifts in specific immune activity among effective molecular weight globulin fractions were found to occur in the salmonid sera during extended periods of immunization. Some consistent but seemingly nonspecific activity was also found in the prealbumin fraction of the trout serum.

SUMMARY

The ERM bacterium has been shown to be a highly infectious and economically significant pathogen of salmonid fishes, particularly under the stresses of intensive culture. Due to its present limited but expanding geographical distribution, it is of primary importance that preventative measures be taken to limit the further dissemination of the disease. Such preventative measures and treatment should take into account the presented findings concerning the epidemiology and pathogenesis of the
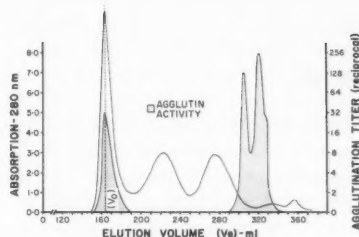
Figure 25.—Gel filtration chromatographic fractionation and passive agglutinin activity determination of hyperimmune rainbow trout, *Salmo gairdneri*, serum proteins.

disease and, in particular, the asymptomatic carrier state of infection. Programs of inspection, certification, and surveillance, based upon presumptive serological screening procedures adapted to large numbers of individual small volume sera with minimum lethal sampling, give promising results for disease diagnosis, surveillance, and detection of clinically inapparent carrier infections.

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Vibriosis and Furunculosis in Marine Cultured Salmon in Puget Sound, Washington

ANTHONY J. NOVOTNY

ABSTRACT—Infections in marine cultured Pacific salmon (genus Oncorhynchus) *and trout (genus* Salmo) *can include those caused by two bacterial pathogens,* Vibrio anguillarum *(vibriosis) and* Aeromonas salmonicida *(furunculosis). In the Puget Sound area, two distinct serotypes of V. anguillarum have caused extensive mortalities in net-pen culture. Although furunculosis is probably carried by fish from fresh water, it can be transmitted in seawater, and in the close confines of net-pen culture can reach epizootic proportions. In large-scale experiments, epizootics of vibriosis and furunculosis reduced the population of two sea cages (300,000) of chinook salmon (O. tshawytscha) by 80 percent during approximately 5 months of marine culture. Laboratory tests of the bacterial pathogen isolated from moribund fish indicated that the furunculosis organism was resistant to oxytetracycline and sulfa drugs, but was sensitive to furazolidone. Multiple infections of both diseases proved difficult to treat. The results of these experiments indicate the need for better management of furunculosis during the freshwater culture stages of salmon.*

Figure 1.—Puget Sound, Washington. This inland arm of the northeastern Pacific Ocean is the site of major activities for the marine culture of Pacific salmon.

Since 1969, the Northwest and Alaska Fisheries Center of the National Marine Fisheries Service has been conducting research on the marine culture of salmonids (*Oncorhynchus* sp. and *Salmo* sp.) at its Aquaculture Experiment Station near Manchester, Wash. (Fig. 1). The major research effort at this station in central Puget Sound is focused on the culture of coho, *O. kisutch,* and chinook, *O. tshawytscha,* salmon in floating net pens.

The principle of this type of culture is the same as for an agriculture feedlot: the fish are concentrated to minimize space, materials, and labor, and are fed commercial pelleted rations. Tidal currents provide an almost continuous exchange of water through the knotless nylon net pens, insuring adequate supply of dissolved oxygen to the fish and the dilution and removal of excretory waste products.

The normal procedure is to transport unacclimated juvenile chinook or coho salmon from a freshwater hatchery and place them directly into seawater pens. The fish are then cultured for 6 months to a year prior to harvesting. The

physiological stresses caused by direct transfer from fresh water to seawater, high population densities, repeated handling, and increasing water temperatures during the summer are conducive to diseases (Wedemeyer, 1970; Wedemeyer and Wood, 1974).

During an experiment to determine the effects of rearing densities on the growth and survival of chinook salmon in seawater pens, repeated epizootics of vibriosis and furunculosis were encountered. The objectives of this paper are to: 1) present a history of these diseases during the course of the experiment; 2) demonstrate drug resistance in furunculosis; 3) demonstrate the infectious nature of furunculosis in seawater; and 4) show that the presence of multiple infectious agents can be a serious threat to marine cultured salmonids.

DESCRIPTION OF VIBRIOSIS AND FURUNCULOSIS

The two most common diseases occurring in salmon cultured in seawater in Puget Sound are vibriosis and furunculosis. Both of these bacterial diseases can be either epizootic or chronic; they
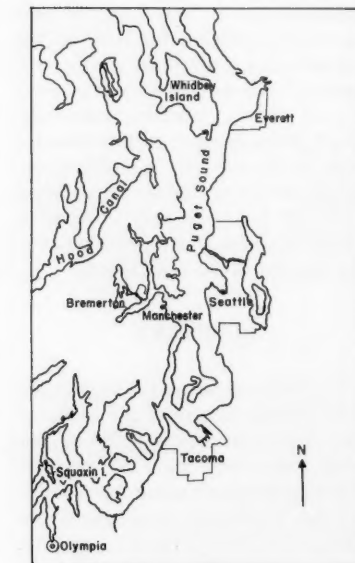
can also occur in marine fish other than salmon (Evelyn, 1971a, b; Kennedy, 1974; Egidius and Andersen, 1975; Novotny, 1975). The typical symptoms of both diseases are almost identical: a general hemorrhagic septicemia, external lesions, hemorrhaging of the fins, and bloody discharges from the vent.

Vibriosis in fish is an infection caused by the marine bacterium *Vibrio anguillarum* (Bergman, 1909), a motile, gram-negative rod which has been thoroughly described by Evelyn (1971b). At least two pathogenic serotypes have been identified (Novotny, 1975; Harrell et al., 1976).

Furunculosis in fish is an infection caused by the nonmotile, gram-negative rod, *Aeromonas salmonicida* (Lehmann and Neumann, 1896). This disease has been described (McCraw, 1952; Scott, 1968), but is generally considered to be a serious problem in freshwater environments only. Unlike

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the vibrios, *A. salmonicida* is not considered to be a marine organism. In salmonids, the disease is undoubtedly first contracted during the freshwater culture stages. At low temperatures, the disease is latent and in a carrier state in infected fish (Snieszko, 1969). When the fish are transferred to seawater, the combined effect of osmotic stress and other environmental factors weakens the hosts, and the disease become infectious. Transmission in seawater can undoubtedly occur by direct contact (Scott, 1968) and probably by contact with fecal casts.

DISEASE OBSERVATIONS DURING 1972-73

Severe outbreaks of vibriosis and furunculosis among chinook salmon during an experiment to determine the effect of different fish densities on fish growth and survival in pens allowed extensive observation of the diseases. Observations on diagnosis, drug sensitivity tests, treatment, and mortality assessment are mentioned below.

In June 1972, juvenile chinook salmon were transferred from trucks to floating net pens by gravity flow through a large pipe. The fish went directly from the fresh water in the transport trucks to 30‰ seawater. The floating hexagon-shaped pens, made from knotless nylon webbing, measured 4.9 m on a side, were 3.8 m deep, and had an approximate volume of 220 m³.

One hundred thousand salmon, weighing an average of 5.4 g each were placed in Pen I and 200,00 in Pen II. The temperature of the water in the transport truck was 13.9°C for Pen I fish and 17.9°C for Pen II fish. The temperature of the seawater was 11.2°C. The fish were to be reared in the pens for approximately 1 year, during which time they were to be fed a daily ration of Oregon Moist Pellets (OMP), based on a percentage of body weight.

Diagnostic procedures

Sick or freshly dead fish were routinely examined for disease. Samples of kidney tissue were aseptically streaked onto Petri dishes containing

Trypticase Soy Agar (TSA)[1] with 1.5 percent NaCl or Tryptose Blood Agar (TBA). Sterile discs impregnated with the vibriostatic agent 2,4-diamino-6,7-di-iso-propyl pteridine phosphate (0/129) were placed on freshly streaked TSA plates. All plates were incubated at 24°C for 24 to 72 hours. A diagnosis of vibriosis was based on: 1) growth of smooth, opaque colonies in 24 to 48 hours on TSA, 2) motility, and 3) a zone of inhibition to the 0/129 disc of 8 mm or more. Furunculosis was considered to be the causative agent of disease when: 1) Pin-point colonies appeared within 72 hours on TBA, 2) the organisms were nonmotile, and 3) the TBA plates would be pigmented a light to chocolate brown.

Because of the large quantities of diseased fish that were processed, it was impossible to confirm all cultures by biochemical reactions. However, culture samples were periodically analyzed by the Department of Microbiology, University of Washington, for confirmation. In general, the accuracy of the field diagnosis was high.

Drug Sensitivity Tests

Drug sensitivity tests were conducted by using discs containing 30 μg of oxytetracycline or 100 μg of furazolidone placed on freshly streaked TSA or TBA plates. Triple sulfa (SSS) was tested by placing discs containing 250 μg SSS on freshly streaked plates containing Mueller-Hinton's agar. All plates were incubated at 24°C until sufficient growth occurred to indicate zones of inhibition. A zone of inhibition of less than 10 mm indicated a questionable effective value.

Therapeutic Treatment Procedures

All of the fish were placed on OMP-TM-50D (OMP-TM-50D is a commercial pellet containing 4 percent TM-50D; eleven percent of TM-50D is active oxytetracycline) medicated diets for the first 5 days after transfer as a precautionary measure to assist in combating any stress infection induced by transfer shock. Routinely thereafter,

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

whenever the mortalities reached 0.1 percent/day and vibriosis or furunculosis was determined to be the cause, OMP-TM-50D diets were fed the fish for at least 5 days, or until the mortalities subsided to less than 0.1 percent/day. When it became evident later in the study that the furunculosis organism was resistant to oxytetracycline, standard OMP diets were prepared daily with commercial agriculture grade Furox 50. The Furox 50 (which is 50 percent active furazolidone) was mixed with herring oil and sprayed over the pellets at a rate of 2 percent oil by weight. The amount of Furox 50 added to the oil was adjusted to provide 0.8 g of Furox 50/kg fish per day.

Mortality Assessments

Dead fish were removed from the pens each day and counted, except when mortalities were excessively high. In this case, total weights were taken, and an estimate of mortality was obtained from subsample weights and counts. Subsamples of the live fish population were obtained periodically to measure growth rates and to adjust feeding levels.

RESULTS AND DISCUSSION

The initial 10-day mortality in the high density pen (Pen II) was 0.5 percent compared to 0.1 percent in the low density pen (Pen I). This was believed to be due to the greater temperature shock during the transfer of Pen II fish.

Within 3 weeks of transfer, the nets became heavily fouled with sessile filamentous algae, reducing water flow through the pens. Dissolved oxygen concentrations in the center of the pens occasionally dropped to 3.5 ppm, creating an oxygen stress condition. This condition was somewhat alleviated by forcing large volumes of compressed air through perforated pipes beneath the pens. The rising air bubbles brought in new water, creating a partial exchange. This problem was solved in late July, when larger mesh net pens were installed.

Water temperatures reached 12°C early in July (Fig. 2). At this time, the first serious epizootic of vibriosis occurred. At times, the mortalities were
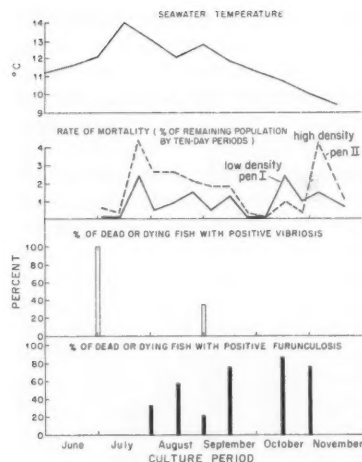
Figure 2.—Incidence of vibriosis and furunculosis in addition to estimated rates of mortality in the high and low density pens in relation to season and seawater temperature. Note that the declining seawater temperature in the fall did not inhibit an epizootic of furunculosis.

so numerous that it was difficult to estimate mortality rates or standing populations. Deterioration of dead fish was so rapid that many were never recovered. Therefore, 10-day mortality rates in Figure 2 are based on estimates of the standing population. Mortality rates were generally in excess of 1 percent (≥ 0.1 percent/day), and the rate of mortality in Pen II was always higher than in Pen I, with the exception of a period in October (Fig. 2).

Our procedure for treatment, based on past experience, was to use OMP-TM-50D exclusively to combat vibriosis. Normally, an OMP-TM-50D diet was only necessary for a period of 5-7 days to control the disease. Rarely had oral medication been used more than three times during a growing season (summer-fall). However in this study, the mortalities were so numerous that we doubted we could control the disease with medicated feeds.

Figure 2 clearly indicates that the dominant problem was furunculosis. When the first epizootics of furunculosis appeared in July, we began testing cultures immediately. We found that the furunculosis organism was: 1) Resistant to oxytetracycline (whereas

Table 1.—Salinity tolerance and antibiotic sensitivity of *Aeromonas salmonicida*[1].

Sample no.	I	II	III	T_{30}	SSS	F_{100}
1	+ D	+ M	− N	0	11	25
2	+ D	+ M	− N	0	8	12
3	+ D	+ L	− N	0	7	15
4	+ D	+ D	− N	Not conducted		
5	+ D	+ M	− N	0	11	22
6	+ D	+ L	− N	Not conducted		
7	+ D	+ M	− N	0	5	12
8	+ D	+ M	− N	0	0	10
9	+ D	+ M	− N	0	5	20
10	+ D	+ M	+ N	0	0	9
11	+ D	+ D	− N	0	0	15
12	+ D	+ M	+ N	0	0	15
13	+ D	+ M	+ L	0	0	15
14	+ D	+ M	− N	0	0	12

[1]Tests were performed with direct isolates from kidney smears on Tryptose Blood Agar and Mueller-Hinton's Agar. Samples collected 1-2 November 1972. I = 1.5 percent NaCl; II = 2.0 percent NaCl; III = 3.0 percent NaCl. + = good colony growth; − = no growth. Agar pigmentation: D = dark; M = medium; L = light; N = none. Antibiotic sensitivity discs (distance is the radius in mm): T_{30} = 30 μg oxytetracycline; SSS = 250 μg triple sulfa; F_{100} = 100 μg furizolidone.

the vibrio was not) and triple sulfa compounds, but sensitive to furazolidone, and 2) it would grow on media containing between 2 and 3 percent NaCl (Table 1). This second finding indicated that this strain of furunculosis might be transmittable in seawater. Later, we isolated this organism from adult coho salmon cultured in pens within a 15-mile radius of Pens I and II. In addition, we isolated the organism from marine fish that accidentally entered the pens with the infected chinook salmon (Novotny, 1975).

Furox 50 was tried as a treatment for furunculosis. The first Furox 50 treatment period was extended to approximately 12 days in an attempt to reduce the numbers of low-level infectious or carrier fish as much as possible. We assumed that once the temperatures started dropping in early fall, our problems of recurring epizootics would be over. The rate of observed mortality declined through August, and a second treatment in August reduced the mortality rate to an even lower level in September (Fig. 2). However, by mid-October, the rate of mortality again began to climb in spite of decreasing water temperatures. *Aeromonas salmonicida* was isolated from almost all subsamples of dead or dying fish.

In late October, we inventoried the high density pen by passing all of the
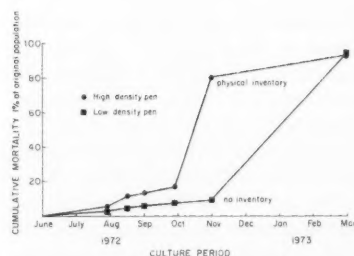
Figure 3.—Estimated cumulative losses of chinook salmon in the high and low density pens. The large disparity between the two pens in November is an estimating error. The high density pen was physically inventoried, whereas the low density pen was not inventoried until the following March.

fish in the pen through a photocell fish counter. In spite of Furox 50 feedings before and after inventory, over 7,000 mortalities were recorded within 10 days. The inventory indicated a cumulative loss of 80 percent of the fish in Pen II (Fig. 3). Although the data in Figure 3 indicate a better survival in Pen I in November, this is an unreliable estimate, and the cumulative mortality was more likely between 70 and 80 percent. We continued the Furox 50 treatment in both pens periodically through the winter.

By February 1973, the surviving fish in both pens appeared to be in good enough condition to inventory. The fish were weighed and samples collected to determine average weights. We estimated only 6,000 fish surviving in Pen I and 14,000 in Pen II, for a total survival of 6 and 7 percent, respectively. At no time during the course of the experiment did we ever reach a planned high loading density of 32 kg/m^3 (Fig. 4).

This total mortality of 93.5 percent is contrasted with a loss attributed to vibriosis of 3.2 percent during the 1971-72 rearing period at Manchester and a 1972-73 loss of 15.7 percent (directly attributable to vibriosis) of a different stock of chinook salmon (Moring, 1973). These lots represented stocks of chinook salmon from other hatcheries, no incidence of furunculosis was found, and all epizootics of vibriosis were easily controlled with OMP-TM-50D.

When the growth of the fish in the
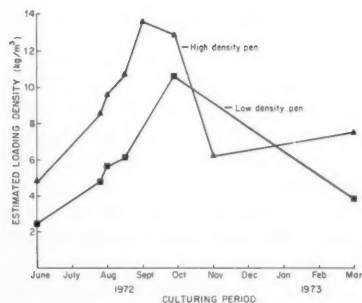
Figure 4.—Estimated loading densities of chinook salmon in the two pens during the spring-winter culturing periods. No physical inventory of the low density pen was taken in November, and the fall estimate of this pen is probably in error.
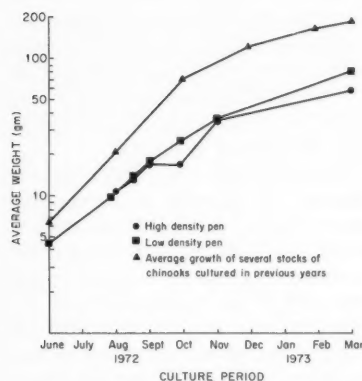
Figure 5.—Growth of chinook salmon in the density tests. A comparison is made with average growth rates for previous years.

loading density study was compared with the average growth of several stocks of chinook salmon cultured in previous years (Fig. 5), there is a depression in the rate of growth in the density of fish. Moring's (1973) average fish weights were 145 g in February 1972 and 92 g in February 1973. By contrast, the average weights of the Pen I and Pen II chinook salmon in February 1973 were 80 g and 59 g, respectively.

We felt that we had been unsuccessful in combating the infectious diseases for three primary reasons: 1) Environmental stress caused by low dissolved oxygen, 2) a carrier state of furunculosis, and 3) high loading densities. Secondary factors were handling stres-

ses and the fact that the average size of the juvenile chinook salmon that were transferred to the net pens was considered to be too small for successful adaptation to saltwater. (Juvenile chinook salmon should average at least 8 g when transferred to seawater.) Osmotic stress is an important factor in predisposing fish to disease (Wedemeyer, 1970; Wedemeyer and Wood, 1974).

Approximately 20 percent of the fish were tagged, and the entire population was released between May and July 1973. The fish that were released were apparently healthy survivors. On the basis of actual tag recoveries, Moring (1973) estimated a 0.1 percent contribution to the sport fishery, and contributions from the tag recoveries of the released density-study fish were 0.3 percent. However, neither of these contributions is high. Moring (1973) notes that chinook salmon reared for 9 months in pens at Manchester and released in April 1971 had a 12 percent recovery rate in the sport fishery—a recovery 40 times greater than that of the fish used in the density study.

CONCLUSIONS

1) Juvenile chinook salmon are highly susceptible to vibriosis and furunculosis when subjected to excessive physical, environmental, and/or osmotic stress following transfer to seawater culture systems. Seawater temperatures in excess of 12°C contribute to the severity of epizootics of vibriosis and furunculosis. Epizootics of furunculosis recurred in the fall, in spite of declining seawater temperatures.

2) Furunculosis can be carried by the host fish in a latent state from fresh to saltwater. Furthermore, the growth of furunculosis on culture media containing 20-30‰ salt and other evidence suggests that the organism may be transmitted in saltwater.

3) Drug sensitivity tests demonstrated that the strain of A. sal-

monicida isolated in this study is resistant to oxytetracycline and sulfa compounds but sensitive to furazolidone. Severe combined epizootics of vibriosis and furunculosis could not be effectively controlled by feeding medicated diets containing oxytetracycline.

4) These observations suggest that a serious effort must be put forth to control furunculosis in fresh water.

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Parasitic Diseases of Freshwater Fishes

W. A. ROGERS

ABSTRACT — The major parasites of freshwater fishes are briefly discussed. Life cycles, host-parasite relationships, pathogenicity, and possible prevention and control are given.

Parasitic diseases of fishes are usually encountered more often then microbial diseases. From 30 to 50 percent of the cases received at several fish disease diagnostic laboratories involve parasites.

Host reaction to parasitic invasion is highly variable. The severity of a parasite epizootic may be related to environmental factors; host condition, age, and size; and population density. Some species that may be considered commensals become pathogenic under certain conditions. However, most obligate parasites are pathogenic and some, such as the ciliated protozoan *Ichthyophthirius*, cause great losses. Many helminths, such as the digenean grubs, appear to be only slightly or moderately pathogenic but destroy the aesthetic value of fish.

PROTOZOANS

Many biologists consider the protozoans to be the most important group of parasites affecting fish. Fish culturists throughout the world report great losses caused by protozoans. The most devastating fish parasite of all is *Ichthyophthirius*. "Ich" is an obligate parasite infecting the skin and gills of fish. The life cycle of "Ich" involves production of many young individuals (tomites) produced from a single cell after the mature cell, the trophozoite, leaves the fish. The trophozoite settles on a substrate, secretes a cyst wall and undergoes multiple fission, giving rise to up to 1,000 tomites. The tomites then leave the cyst, seek a host, bore into the epidermis or gill epithelium, and produce a severe irritation accompanied by excess mucus production and hyperplasia of the epithelium. The cells mature, creating small white spots that are easily seen with the naked eye.

We were able to demonstrate that fish will develop an immune response to *Ichthyophthirius* infections. After fish were experimentally infected, they were treated with malachite green to rid them of "Ich". Fifteen days later they were challenged: All control fish developed "Ich" and died while previously infected fish did not.

One researcher collected "Ich" trophozoites, ground them up using 1.0 ml of trophozoites in 10 ml of 0.85 percent saline, added Freund's adjuvant, and injected the fish intraperitoneally. Fifteen days later upon challenge the controls developed "Ich" and died, but all injected fish survived (Areerat, 1974). A problem in developing a vaccine for "Ich" would be in-vitro culturing of "Ich." Many people have attempted in-vitro culture without success.

Several other ciliates such as *Chilodonella*, *Trichodina*, *Ambiphrya*, and *Epistylis* can cause mortality under certain conditions. Flagellates such as *Costia* and *Bodomonas* often cause mortality but are fairly easy to control with an external treatment.

Spore-forming Cnidospora include members of the genus *Myxosoma* that cause whirling disease of salmonids in which young fish are infected and the cartilage destroyed prior to bone formation. When the eroded cartilage ossifies, the bone is distorted and the fish usually has severe curvature of the backbone, resulting in the fish swimming in a circle thus giving the condition the name "whirling disease."

Henneguya is another Cnidosporidian commonly infecting channel catfish, *Ictalurus punctatus*. Several different species occur in the gills and skin with an interlamallar form reported to cause extensive mortality in very young fish.

Several species of *Pleistophora*, a microsporidian, occur in fish, one of which occupies the ovary of golden shiners, *Notemigonus crysoleucas*, and can cause sterility. Older fish have a much heavier infection rate and more ovary damage, but a technique of using 1-year-old brood fish has practically eliminated the problem of sterility.

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TREMATODES

Some genera of monogenetic trematodes can build up large populations under intensive culture situations but they are not considered to be a serious threat to fish in North America.

Two groups of digenetic trematodes occur in fish: adults normally living in the gastrointestinal tract and larval forms, metacercaria or grubs, living in the tissues. The eye grub, *Diplostomum,* attacks the lens of the eye causing blindness while white grubs, black grubs, blackspot, and yellow grubs affect mainly the aesthetic value of the fish. There are no controls known for the tissue-inhabiting larval forms.

CESTODES

Cestode pleurocercoids of the largemouth bass, *Micropterus salmoides,* tapeworm, live in visceral organs of bass and commonly migrate through ovaries causing sterility. One approach we have taken is replacement of brood fish about every 3 years in an attempt to manage around the problem by maintaining a low intensity of infection. Earlier attempts to rid fish hatcheries of the bass tapeworm by disinfecting the ponds and restocking both brood and forage fish were unsuccessful as the fish would show a reinfection within a year after restocking. *Corallobothrium* is a tapeworm sometimes found in great numbers in the intestine of channel catfish. Little apparent damage is caused by this worm and it has been easily controlled using tin compounds in the feed.

NEMATODES

Nematodes in the gastrointestinal tract are not considered detrimental, but tissue inhabiting forms can seriously affect the fish population. *Philometra* is a nematode commonly found behind the eye of centrarchid fishes and up to 40 percent of some reservoir populations in the southeastern United States have been affected.

CRUSTACEANS

Crustaceans are one of the main groups of detrimental parasites, especially the anchor worm that has both adult and a parasitic larval stage that feeds on the fish.

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MFR PAPER 1299

Ornamental Fish: Diseases and Problems

JOHN B. GRATZEK, EMMETT B. SHOTTS, and JACK L. BLUE

ABSTRACT—*Seventy-seven bags of ornamental fish imported from Hong Kong, Taiwan, Singapore, and Bangkok were examined for parasites, bacteria, and viruses. Parasites exotic to native North American fishes were not found. Eighteen genera of bacteria were identified. One virus, tentatively classified as a herpesvirus was isolated from a pooled sample of macerated Kuhli loach,* Acanthophthalmus *sp., tissue.*

Approximately 600 million tropical fish are imported into the continental United States each year. The objective of this study was to evaluate the potential ecological impact which these fish and/or the water in which they are shipped could have on the health of humans, domestic animals, or native fish species. In this study, 16 shipments of tropical fish originating in Hong Kong, Taiwan, Singapore, and Bangkok were examined for presence of parasites, bacteria, and viruses; 77 bags of fish were examined.

METHODS

Parasitological Examinations

Five fish from each bag of fish were examined following standard methods (Reichenbach-Klinke, 1973) of dissection and examination. Wet mounts of gills, skin and fin scrapings, and internal organs were examined using 5 and 10 magnification objectives.

Bacteriological Examinations

Upon arrival, the fish and their shipping water were systematically processed for the presence of bacterial flora. The bags were opened and a sample of

fish removed for parasitic studies. From the remaining fish in each bag, five randomly selected individuals were cultured for detection of possible bacteremia. This was accomplished by killing and surface sterilization of the fish with aseptic dissection to reveal the appropriate organ (kidney) for culture. In small fish a mixture of blood and/or kidney was cultured.

An additional sample of fish was killed, surface sterilized, and homogenized in a blender with sterile phosphate buffered saline at pH 7.2 to achieve a 10 percent weight/volume suspension of fish.

Concurrent with fish processing, aliquots of water from each bag were pooled to achieve approximately a 1,500-ml sample of water representative of the respective shipment. This sample was centrifuged at 5,000 RPM for 20 minutes in an RC-2B[1] centrifuge at 4°C.

The above samples were cultured as follows:

1) Blood and/or kidney were streaked on blood agar and Ordall's

agar with subsequent incubation at 35°C and 23°C. Resulting growth was subcultured and identified.

2) A battery of several media was inoculated with fish suspension to assure recovery of a wide spectrum of bacteria. Selective media were used where possible to enhance possible isolations. The media used and justification are: A) Blood agar was used as a general medium for detection of fastidious organisms as well as common organisms; B) Ordall's agar was selected for detection of the presence of myxobacteria; C) Trypticase soy agar was used as a generalized media which would grow practically all organisms: D) Rimler-Shotts agar (Shotts and Rimler, 1973) was used to specifically select members of the *Aeromonas hydrophila* complex; E) Bismuth sulfide agar was used specifically to detect the possible presence of *Salmonella typhosa;* F) Selenite-brilliant green broth and dulcitol selenite broth—brilliant green agar. This battery of media was used for the detection of possible *Salmonella.*

Unless otherwise necessary because of special requirements of the media, all media were incubated at 35°C and 23°C. Resulting growth was subcultured and identified by standard methods (Breed et al., 1957).

3) Water. The sedimented material

[1]Reference to trade names or commerical firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

resulting from centrifugation was resuspended in approximately 100 ml of the centrifuged supernatant. This constituted the inoculum for the following battery of media: A) Selenite brilliant green broth and dulcitol selenite broth—brilliant green agar. This battery of media was used for detection of possible *Salmonella;* B) MacConkey agar was used to detect gram negative bacteria and also group them for further processing; C) TCBS—was used as a selective medium for *Vibrio cholera* or *Vibrio parahemolyticum* which might be present; D) Trypticase soy agar with 1.5 percent sodium chloride was used for detection of possible halophilic organisms; E) Rimler-Shotts agar was used for selection of members of the *Aeromonas hydrophila* complex; and F) Pseudosel was used as a selective medium for the genus *Pseudomonas.*

These media were incubated at 35°C and 23°C unless otherwise indicated by the selective procedure involved. Resulting growth was subcultured and identified. Aliquots of all samples were processed on special media for the presence of *Mycoplasma* sp. and *Mycobacterium* sp.

Virological Examinations

Samples were processed for virus isolation by making 10 percent suspension of whole fish in phosphate buffered saline. The suspension was filtered through four layers of cheesecloth and centrifuged at 1,000 g for 15 minutes. The supernatant was removed and recentrifuged at 4°C at 5,000 g and then filtered through a 47-μm pore size membrane filter using positive pressure.

Cell cultures used for virus detection were rainbow trout gonad (RTG-2), fathead minnow (FHM), brown bullhead (BB), VERO (African green monkey kidney), pig kidney, bovine kidney, rabbit kidney, and feline kidney. Medias, cell culture procedures, and microculture techniques have been described previously (Gratzek et al., 1973; Rovozzo and Burke, 1973). All samples were passed three times at 5-day passage intervals. Samples were judged negative if cytopathic effects

Table 1.—Gill infestation.

Parasite	Bags infested %
Flukes	21.0
Ichthyophthirius	2.6
Flukes + *Ichthyophthirius*	1.3
Trichodina + *Ichthyophthirius*	1.3

Table 2.—Skin infestation.

Parasite	Bags infested %
Flukes	3.9
Chilodonella	1.3
Oodinium	1.3
Ichthyophthirius	1.3
Myxosporida	1.3

Table 3.—Intestinal infestation.

Parasite	Bags infested %
Nematodes (in lumen)	12.5
Nematode (cysts)	6.2
Acanthocephalan nematode (cysts)	3.1
Acanthocephalan (cysts)	3.1
Hexamita	3.1

were not present at the end of the third passage.

RESULTS AND DISCUSSION

Parasitological Results

The results of parasitological examinations of 77 bags of fish suggested that 61 percent contained fish with some type of parasite. In 39 percent of the bags, no parasites at all were found. The results are best seen in Tables 1, 2, and 3, which present the incidence of fish parasitized.

Gill flukes were the commonest parasites observed on these fish. However, it is also important to note that only in one case was the fluke infestation high enough to create immediate problems for the fish. Also, it appears that combinations of infections, such as flukes and *Ichthyophthirius,* or *Trichodina* and *Ichthyophthirius* do occur.

The results of intestinal examination for parasites suggested that 12.5 percent of the 77 bags contained fish that had nematodes in the interior of the intestine. The significance of cysts of nematodes or acanthocephalans found

imbedded in tissues outside the intestine either alone or in combination is that these fish are most probably intermediate hosts for these parasites where the adult stages of the worms exist in birds or larger fishes. Heavy infestations of these intermediate forms are harmful to these fish. It is significant to note that similar families of parasites are found in fish native to this country. It has been stated (Meyer, 1954) that under conditions in nature there is rarely a single individual fish among all the numerous species from the smallest minnows to game fishes which does not harbor at least one or more species of parasites somewhere in its body. Often the parasites are confined to the internal organs and hence are usually not noticed when the fish is cleaned or dissected. It would appear then that the incoming fish are possibly parasitized certainly no more than native fish species.

We were surprised to note that these fish did not harbor more intermediate forms of digenetic flukes or tapeworms. A possible explanation is that most of the fishes imported from the Far East are raised in aquaria or in small ponds where there is less chance for infestation of these fish by free swimming intermediate forms of these parasites. Preliminary studies from South American fishes suggest that the opposite is true.

Bacteriological Results

In this study, 77 bags containing 30-50 fish each were examined. In general, the bacteria isolated were primarily rod shaped, gram negative staining types which, in most instances, can be associated with the fish's natural environment. Bacteremias were noted in fish in 51 bags and represented 11 genera of bacteria. This high incidence of bacteremia suggests that fish are under severe stress during shipment. Further detailed bacteriological cultures of each lot of fish resulted in the isolation of 18 genera of bacteria. Examination of the water in which the fish were shipped resulted in the isolation of 14 genera of organisms. More prevalent among the

bacteria isolated were those of the genera *Pseudomonas, Aeromonas, Proteus, Citrobacter, Enterobacter,* and *Escherichia.* The first two, while they may be potential fish pathogens, are considered normal inhabitants of water and constitute no disease problem to mammals or fish under normal conditions. The latter organisms are usually indicative of human or other animal association and while occasionally associated with human or animal disease are not considered of public health importance under normal circumstances.

Only two organisms which could be considered of human health importance were recovered during the study. These organisms were *Salmonella arizona,* which in high numbers may cause human diarrhea, and *Mycobacterium* sp., which are universally found in water. The former was recovered from fish and water in one instance each. The latter was recovered from the water on three occasions. *Mycoplasma* sp. were not isolated.

Virological Results

One virus isolate was made from a slurry of Kuhli loachs, *Acanthophthal-mus* sp., and from the water in which these fish were transported. The virus was isolated on rabbit kidney cell cultures. The virus was characterized as a herpesvirus based on size and morphology, DNA content, ether susceptibility, and lack of hemagglutination ability. The virus was shown to not cross-react with channel catfish herpesvirus nor was it pathogenic to channel catfish, *Ictalurus punctatus.* It does not react with antiserums to pseudorabies virus or infectious bovine rhinotracheitis virus, but it does partially cross-neutralize with equine rhinopneumonitis virus. Further serological studies are being conducted.

SUMMARY AND CONCLUSIONS

The results of this survey of imported fishes from Southeast Asia indicate that the parasitic load is less than would be expected in native fish (Hunter, 1942). The presence of bacteria of definite public health importance is also minimal based on reported studies on *Salmonella* distribution in continental U.S. watersheds (Kenner and Clark, 1974). The isolation of a herpesvirus, so far not completely characterized, supports the observation that viruses, like bacteria, are found in water from various sources.

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Shellfish Diseases

LOUIS LEIBOVITZ

ABSTRACT—An overview of commercial bivalve shellfish aquaculture is presented. The advantages and disadvantages of shellfish production as compared with other forms of food animal production is discussed. The common shellfish diseases are listed and the known specific etiologic agents are indicated. The latter include viral, bacterial, fungal, protozoan, and metazoan parasitic and infectious agents. In addition, predators, toxic agents, and fouling organisms produce serious economic losses.

The specialized problems of shellfish hatcheries are discussed. The importance of monitoring the qualitative physical, chemical, and bacteriological changes in shellfish larval cultural media and its ingredients for optimum production is indicated.

A description of a laboratory model for evaluating the pathogenicity of pure bacterial cultures for larval shellfish is presented. The experimental optimal and lethal concentrations of bacteria for shellfish larvae are defined. An interrelationship between bacteria and protozoa in the pathogenesis of shellfish larval diseases is reported. The shellfish industry has encouraged and supported the reported research to increase the efficiency of shellfish production by reducing economic losses due to shellfish diseases.

Less is known about the subject of shellfish diseases, and, accordingly, there is a wider latitude in discussing it. There are many unique problems, some of which overlap with fish diseases.

One problem is that the molluscan bivalves are filter feeders. Their ability to concentrate harmful chemicals and infectious agents pose serious problems in controlling both shellfish and human diseases. Since shellfish are estuarine dwellers, they are subjected to environmental variations such as changes in salinity and temperature, seasonal tidal variations, and varying degrees of

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exposure to urban and industrial pollutants discharged into estuarine waters.

In spite of these hazards, shellfish hold one of the greatest potentials for the economic production of food protein. Shellfish hold great promise for the efficient recycling of organic waste materials, such as agricultural wastes, and the capture of energy for food production from thermal effluents, such as that discharged from atomic power plants. In addition, there are more species of shellfish than any other group of animals, with the exception of arthropods. Genetic selection for greater food yields from these abundant varieties should be rewarding. Also, in terms of reproductive potential, there are no other food animals that even approach their fecundity. For example, a single pair of oysters can produce as

many as 120 million offspring from a single mating.

There is another unique aspect of shellfish production that exceeds the economic efficiency of other forms of animal protein production and that is free food. Unlike the rising food costs of other animal feeds, shellfish foods are naturally generated planktonic foods. Since shellfish are an important source of food, we should learn more of their diseases as a part of the technical development necessary to increase production.

The following discussion of shellfish diseases is an overview and a short consideration of one specific bacterial disease problem in larval shellfish production being currently studied.

DISEASES OF SHELLFISH

A list of organisms that cause common diseases in oysters is shown in Table 1.

Viral Diseases

Of the known reported virus infections of oysters, "Ovacystis" infection is the most common, but it is probably of little economic importance. It can be detected histologically as hypertrophy of the ovarian follicles. The affect of this virus upon reproductive performance has not been evaluated.

A herpes virus infection has been described by Austin Farley (1972) in oysters. Apparently, expression of the disease was temperature dependent and was found in oysters cultivated in the heated effluent of a power plant. When

Table 1.—Causes of common diseases in oysters.

Group	Species
Viral	Ovacystis virus
	Herpes virus
	Others
Bacterial	Achromobacter sp.
	Aeromonas sp.
	Vibrio sp.
	Mytotomus ostrearum
	"Maladie Du Pied"
	Nocardia sp.
	Cladothrix dichotoma (Actinomycetes)
	Others
Fungal	Dermocystidium (Labyrinthomyxa)
	marinum
	Sirolpidium sp.
	Others
Parasite	1. Sarcodina (Amoeba)
protozoan	Flabellula sp.
	2. Mastigophora (Flagellate)
	Hexamita nelsoni
	3. Sporozoan
	a. Gregarine
	b. Haplosporidia
	4. Ciliates—many
Helminthic	1. Trematoda (larval)
	Bucephalus sp.
	Others
	2. Cestoda (larval)
	Tylocephalum sp.
	Others
Arthropods	1. Copepods
	Mytilicola intestinalis
	Others
	2. Decapods
	Pinnotherid crabs
	3. Annelids ("Mud blisters")
	Polydora websteri
	Others
	4. Sponges ("Boring sponges")
	Clinona celata
	Others

the environmental temperature dropped, the disease was not apparent. While there are undoubtedly other shellfish viral diseases present, they have not been defined.

The two previous viral diseases mentioned were demonstrated upon the basis of diagnostic inclusion bodies and electron microscopic demonstration of viral particles in affected cells. Virus-free molluscan tissue culture systems are needed to isolate and identify molluscan viruses and human viral pathogens that may be carried by shellfish.

Bacterial Diseases

Little is known of the bacterial diseases of shellfish, and the list in Table 1 is limited to those that have been described. From the standpoint of human health, outbreaks of cholera have been related to the consumption of shellfish in Africa and Italy.

Fungal Diseases

Dermocystidium (*Labyrinthomyxa*) *marinum* is a very important shellfish

pathogen that produces serious economic losses in adult shellfish in warm climates. *Sirolpidium* sp. is a common infection of hatchery-reared larval shellfish.

Helminthic Diseases

Among the helminth parasites of shellfish, trematode, cestode, and nematode parasites may be found. Larval forms of trematodes (especially *Bucephalus* sp.) and cestodes (especially *Tylocephalum* sp.) are of economic importance as shellfish pathogens that often produce sterility in affected shellfish. Most of the larval forms mature in fish which serve as definitive hosts. Some are of public health significance.

Arthropods and Other Organisms

In addition to helminth parasites, copepod crustacean and polychaete annelids, during some stage of their life cycles, may parasitize shellfish with resultant serious economic losses.

A great variety of marine organisms are found in shellfish beds in apparent symbiotic or commensal relationships to shellfish. Some, as pinnotherid crabs, enter and leave the pallial cavity of shellfish freely. Crabs may serve as the intermediate host for the primitive gregarine sporozoans (*Nemotopsis* sp.) whose spores infect shellfish with little resultant tissue damage. Macroalgae and sponges grow on the surface of shellfish. The boring sponges (*Cliona* sp.) may damage the external shell and the shell may then become porous and crumble.

Diseases of Unknown Etiology

In addition to the known diseases, many unexplained die-offs have been reported that have decimated shellfish populations. Often these populations do not recover, and new stock, introduced to repopulate, are quickly affected and die. Such diseases are often named for the locality in which they occurred, such as "Malpeque Bay" and "Denman Island" disease. Often serious losses are attributed to climatic conditions, water quality changes, and

pollution without adequate evidence that disease was not responsible.

Parasitic Diseases

Protozoans

Shellfish protozoan infections are very common. Whether these organisms are primary infectious agents is often questionable. This is especially true of the ciliates that are common inhabitants of shellfish tissues. They become especially active when other pathogens such as bacterial agents are present. Of the flagellated protozoa, *Hexamita* sp. and the amoeboid protozoa are pathogenic. When shellfish are maintained under adverse conditions, such as extreme temperatures, protozoa may actively invade shellfish tissues and produce deterioration or spoilage. These conditions may also be found in "winter-kills" of shellfish where high mortality associated with protozoan infections may be found in sustained low temperature exposures.

Protozoans can be primary shellfish pathogens. The most important single shellfish pathogen that has produced the greatest economic losses to the shellfish industry is a haplosporidian, *Minchinia nelsoni*. This organism has destroyed the great oyster industry of the Delaware and Chesapeake Bays. Haplosporidians are very poorly understood, poorly classified sporozoans, distinct from myxosporidia, or coccidial organisms. Their exact taxonomic position and life cycles are unknown. In addition to the areas mentioned, *M. nelsoni*, commonly called MSX, is present in other geographic locations of the northeastern U.S. coastline. This organism is apparently salinity-dependent. It is seasonal in its incidence. There are many other haplosporidians, of varying pathogenicity found as parasites in a variety of aquatic animals. They are found as hyperparasites in trematodes. These organisms tend to sterilize the trematode host.

SHELLFISH HATCHERY OPERATION STUDIES

When I began working with the Long Island shellfish industry, the problems were overwhelming and it was difficult

to select a single starting point. Perhaps the most important economic problems were based in shellfish hatchery production. If hatchery production could be increased, and livability of larvae and juveniles were improved, restocking and harvesting from shellfish beds would yield greater production and efficiency. The techniques of hatchery operation are well known, but consistent production of healthy larvae is difficult. Shellfish larval disease losses are serious hatchery problems, often of epizootic proportions.

Although specific pathogens were occasionally responsible for such losses, it became apparent that there were many unexplained phenomena associated with the more common losses. In an attempt to resolve these problems, studies of hatchery media were undertaken. These included physical, chemical and microbiological examination of hatchery water supply, stock algal cultures, pooled algal food cultures, and spawn obtained from hatchery breeding stock. Each hatchery operation was distinctive. Some operated all year, others limited their operation to warm weather only. Hatchery water supply was either raw bay water, or from deep saltwater wells. Some operations pumped water into the plant on demand; others held water in large storage tanks that was later gravity fed into the operation on demand. Various methods of screening, filtration, and centrifugation are employed for water clarification. In addition some plants utilize ultraviolet treatment of incoming water, or recycled water for disinfection.

Physical and chemical examination of shellfish larval culture media included measurement of pH, salinity, chemical oxygen demand, suspended and total solids. Other tests including nitrogen determinations are currently being explored. Quantitative counts and identification of dominant bacterial populations of the larval culture media ingredients are also being made.

TESTING BACTERIAL PATHOGENICITY IN LARVAL SHELLFISH PRODUCTION

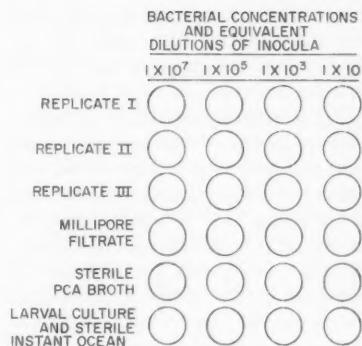It became apparent that a pathogenicity model was needed to test the pure

Figure 1.—Pathogenicity model system to test pure bacterial isolates obtained from larval cultures.
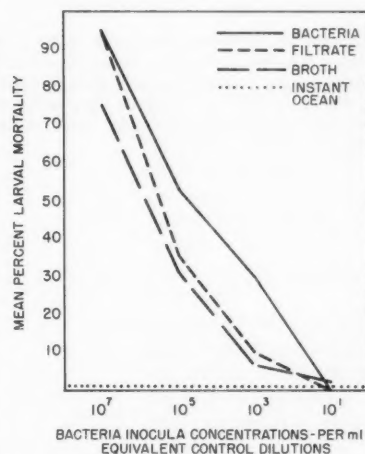
Figure 2.—Graphic representation of mean percent mortality values of equivalent concentrations of inocula of all tested isolates (35) (each replicated in triplicate), their cultural filtrate, uninoculated broth medium, and "Instant Ocean" controls.

bacterial isolates obtained from the larval cultures. The same model could be utilized to test environmental factors, drug efficacy, and other factors for their influence in such disease models. This model system was assembled in plastic "disposo" trays and consisted of 6 rows, of 4 wells per row, containing precalculated approximate numbers of shellfish larvae from 3 to 14 days of age (Fig. 1). To each row was added a

known dilution of the test substance. In testing for bacterial pathogenicity, pure 24-hour broth cultural bacterial isolates were added to each of the first 3 rows of the plate; from left to right, each well of each of the first three rows containing approximately 10^7, 10^5, 10^3, and 10^1 bacteria per milliliter of well larval suspension. The fourth row was given the equivalent dilution of bacteria-free filtrate (Millipore filtrate) of the broth culture to correspond to the dilutions of the bacterial suspension wells above this row. The fifth row received again, equivalent dilutions of sterile culture broth (plate count agar broth - PCA). The last (sixth row) received equivalent dilutions of synthetic sea salts (Instant Ocean)[1] to the shellfish larval suspensions. The results of the above test were read at the end of a 24-hour incubation period. The number of alive and dead larvae in each well was counted and the percentage mortality for each well was determined. In this manner the effects of dilution and comparison of the affects of added ingredients could be measured to determine their relative influence on pathogenicity.

RESULTS

The results of the above pathogenicity tests (Fig. 2) suggest that almost all bacterial isolates at high concentrations ($>10^5$/ml) are pathogenic for shellfish larvae; however, only "true" pathogens kill at very high dilutions ($<10^3$/ml). The latter suggests that these true pathogens require larvae for growth. Note that the presence of higher concentrations of even sterile nutritive broth produces a lethal effect. Accordingly, this may suggest that food concentrations, dead or decaying algal foods, or larvae may aggravate the pathogenic effect of both extrinsic and intrinsic microbial concentration (within the larvae). Future studies are needed. At high concentrations of bacteria and/or equivalent culture media (10^7 or greater), lethal effects are rapid

and are not associated with protozoan activity. However, at levels corresponding approximately to 10^5/ml, lethal effects are indicated by more gradual losses and are associated with intense protozoan proliferation and activity. The latter probably originate from the normal intrinsic microbial flora of the shellfish larvae. In fact, if one were to observe such cultures without knowing of the presence of the experimental bacterial inoculum, the aggressive behavior of the protozoan attack on the shellfish larvae would suggest that they are the primary pathogen. The mechanism responsible for this phenomenon requires further study. Direct observation of the affected larvae in this bacterial study support common diagnostic signs and lesions evident in diseased larvae and is a separate discussion in other studies.

The results of field studies of bacterial populations of hatchery media and its ingredients tend to support the experimental studies. Diseased larval cultures are associated with bacterial populations 10^7 or greater per milliliter. Further studies will be required to define the specific chemical or physical tests of hatchery media and ingredients and their parameters that would be useful in disease detection and diagnosis that could be related to specific pathogenic agents.

As a result of these studies, the need to monitor and define hatcheries for optimum performance becomes more apparent. Since individual hatcheries are different, each hatchery must be evaluated for its operational methods and equipment.

ACKNOWLEDGMENTS

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Control of Fish Diseases

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ABSTRACT — *Severity of outbreaks of communicable diseases of fishes is influenced by environmental conditions. Therefore, beneficial results of chemotherapy depend on the specific action of drugs and the maintenance of conditions favorable to the treated fishes. This paper deals mainly with the therapeutic control of fish diseases.*

RELATIONSHIP OF HOST, PATHOGEN, AND ENVIRONMENT

It is generally accepted that all outbreaks of a communicable disease are the result of interaction between the host, the pathogen, and the environment. This is particularly true in regard to fishes, which are coldblooded and utilize oxygen dissolved in water. The environment of the open sea is very stable, but in the inland waters and in fish farms extreme water temperatures, low dissolved oxygen contents, presence of fish catabolic products, and general pollution often produce stresses which contribute to outbreaks of infectious diseases. According to Seleye (1955), Pasteur, who established the role of microbial pathogens in diseases, allegedly said, "Le microbe n'est rien, le terrain est tout" (the microbe is nothing; the environment is everything).

TREATMENT AND CONTROL OF FISH DISEASES

The practical fish culturist expects the fish disease specialist to provide ironclad remedies which will work under all circumstances (Snieszko, 1975; Fryer, 1978). This is not possible because a drug is just a crutch which is used to help the host survive the infection until the pathogen is subdued and the environment improved. The outcome of treatment depends on the susceptibility of the host to any particular disease, the species, number and virulence of the pathogen, and the degree and duration of stress caused by improper environment (Endo et al., 1973). (See also section on Selected References.)

This relationship can be graphically presented by the use of sets and subsets (Kemeny et al., 1957) as is done in regard to fishes by Snieszko (1973, 1974) and Wedemeyer (1974), and in regard to dental caries by Sherp (1971). It can also be presented in the form of an algebraic equation:

$$H + P + S^2 = D$$

where:
H = species and strain of the host, its age, and inherited susceptibility to any particular disease;
P = the agent causing the disease with all its variability;
S = stress of the environment; and
D = the disease which results if the components on the left side of the equation are in proper qualitative and quantitative relationship.

In this equation, the square of S is used because the stress caused by the environment increases in geometrical progression when the conditions are approaching the limits of tolerance by the host.

Administration of Drugs

Drugs are administered to fishes in a number of ways (Herman, 1970) (Table 1). In external parasitic infestations, drugs can be added to water for different lengths of time. Some antibiotics are injected intraperitoneally. For oral administration, drugs may be mixed with feed. This method is complicated by the fact that the rate of feeding is calculated as a percentage of fish weight. This percentage varies with different fishes and is strongly influenced by the age of fishes and water temperature (Halver, 1972). Therefore, it is often difficult to prepare a diet with just a single concentration of the drug.

Drugs Commonly Used

Drugs which are poorly soluble in water but easily absorbed from the lumen of the intestines are preferable. In case of intestinal parasites, drugs are selected which act in the gut. Many of the drugs used in control of diseases of fishes are the same ones used for humans and domestic animals. Recently, Japanese and German manufacturers released nitrofurans for fishes. They are soluble in water and can be used as baths or mixed with feeds. The Japanese drug is ni-furpirinol (Furanace[1]) (Anonymous, n.d.; Amend and Ross, 1970). The German product is nifurprazine which is sold in Germany as Carofur (Duefel, 1970; Shiraki et al., 1970) and is also licensed for production in Japan as Aivet. These nitrofurans are excellent in systematic infections caused by *Aeromonas punctata* and related forms, and by *Vibrio anguillarum,* and for columnaris disease, gill disease, and others. They are added to water in concentrations from 0.05 to 1 ppm depending on the duration of treatment. These drugs can be used orally with feed. They are quickly eliminated from the tissues leaving no detectable residues within 2 days. Among the older nitrofurans, furazolidone (Furoxone) is effective in oral administration. Among antibiotics most often used are oxytetracycline (Terramycin), chloramphenicol (Chloromycetin), and chlortetracycline (Aureomycin) (Herman, 1970). The latter is used chiefly as a bath for aquarium fishes.

Among the most often used sulfonamides are sulfamerazine, sulfamethazine, and sulfisoxazole (Herman, 1970). Only sulfamerazine and Terramycin are now cleared by the Food and Drug Administration (FDA) for control of certain diseases of fishes.

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[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Chemical agent	Method of administration	Chemical agent	Method of administration
Acetic acid, glacial	Dilute in water: 1:500 for 30-60 seconds (dip) 1:2,000 (500 ppm) as bath for 30 minutes	Formalin (37% by weight of formaldehyde in water. Usually contains 12-15% methanol)	1:500 for 15-minute dip. 1:4,000-1:6,000 for 1 hour. 15-19 ppm to pond or aquarium water for indefinite period.
Acinitrazole (2-Acetamido-5-nitrothiazole)	Used for Hexamitiasis in Norway. 40 mg/kg feed for 4 days	Formalin with Malachite green	In formulations of Formalin with malachite green, Formalin is used at 15 to 25 ppm and malachite green at 0.05 to 0.1 ppm. For several hours in aquaria and for in-
Acriflavine (Trypaflavine)	5-10 ppm added to water from several hours to several days		definite period in ponds.
Aivet (soluble powder contains 6.6% Nifur- prazine HCl (same as Carofur))	See Nifurprazine	Fosfomicina (a Spanish antibiotic; $C_3H_7O_4P$)	For Aeromonas infections in fishes
Aureomycin	See Chlortetracycline	Furanace (P-7138) (Ni-furpirinol); (6-hydroxymethyl-2- pyridine)	Used as bath but may be added to food. As bath: 1 ppm for 5-10 minutes; 0.05-0.1 ppm may be added for indefinite period to water. Orally for treatment: 2-4 mg/kg
Betadine (Iodophor containing 1.0% of iodine)	See Iodophors		fish per day for 3-5 days. Orally for prophylaxis: 0.4-0.8 mg/ fish per day
Bithionol (2,2'-Thiobis) (4,6-dichlorophenol) (a French product also known as Cogla)	Orally 0.2 g/kg of fish or 2% in food. Feed for 2-3 days. For Acanthocephala and oral prophylaxis against Saprolegnia.		as long as needed.
Brilliant green (same as Malachite green G sulfate)	See Malachite green	Furazolidone (Furoxone N.F. 180 N.F. 180 Hess & Clark commerical products contain furazolidone mixed with inert mater- ials)	On the basis of pure drug activity; 25-75 mg/kg body weight per day up to 20 days orally with food.
Bromex (Dibrom, Naled; a pesticide)	0.12 ppm added to (pond) water for indef- inite time.		
Buffodine	See Iodophors Buffodine is a neutral formulation of an iodophor giving nearly neutral solutions in water.	Furoxone	See Furazolidone
		Furpyridinol (product containing 10% Furanace)	Added to water 0.3-10 ppm, as is, for 30-60 minutes. Also see Furanace.
Butyl tin oxide (di-n-butyl tin oxide)	25 mg/kg body weight per day with food for 3 days.	Hyamine (Rohm & Hass Co., quaternary ammon- ium germicide available as crystals or as 50% solution)	1.0-2.0 ppm (on basis of 100% product) in water for 1 hour.
Calcium cyanamide	Distributed on the bottom and banks of drained but wet ponds at a rate of 200 g/m².		
Calcium oxide (quicklime)	Distributed on the bottom and banks of drained but wet ponds at a rate of 200 g/m².	Iodine	In form of a Lugol solution or iodine for control of goiter and possibly corynebac- terial kidney disease.
Carbarsone oxide	Mixed with food at a rate of 0.2%. Feed for 3 days.	Iodophors (Betadine, Wescodyne, Bridine, etc.)	Different commercially available iodophors contain different concentration of io- dine. To be used on a basis of pure io-
Carofur (a product containing 6.66% of Nifurprazine HCl.	See Nifurprazine		dine present in the product. Use 50-200 ppm iodine (usually 100 ppm) for disin- fection of eggs for 10-15 minutes. Toxic to hatched fish. Probably also assist in
Chloramine—I	In water with pH 7.5-8.0, 18-20 ppm. Change 50% of water once each week if water temperature 10°C or below. At 25°C, one treatment for 2-3 days.		control of some virus fish diseases.
Chloramphenicol (Chloromycetin)	1. Orally with food 50-75 mg/kg body weight per day for 5-10 days. 2. Single intraperitoneal injection soluble form 10-30 mg/kg. 3. Added to water 10-50 ppm for indefinite time as needed.	Kamala	Mixed with diet at a rate of 2%. Feed to starved fish for 3 days.
		Kanamycin (antibiotic also traded as Cantrex, Kamycin, Resistomycin)	50 mg/kg of fish or 25-100 mg/kg of food. Feed for a week.
Chlorophos	See Dylox	Malachite green	1:15,000 in water as a dip for 10-30 sec- onds, 1-5 ppm in water for 1 hour; 00.5 to 2.00 ppm in ponds or aquaria for indefinite time.
Chlortetracycline (Aureomycin)	10-20 ppm in water. In eel diseases in Japan it is added to feed at a rate of 10-20 mg/kg of food.		
Ciodrin (Shell Petroleum product; a pesticide similar to Dipterex, Dylox, Masoten).	For control of Lernea in Japanese eel culture.	Malachite green with Formalin	See Formalin
		Masoten	See Dylox
Cogla (D²N Cogla)	See Bithionol	Mefarol (probably similar to Hyamine)	1-2 ppm in water for 1 hour. Toxic in very soft water; less effective in hard water.
Concurat (2,3,5,6 tetrahydro-6-phenylimidazo (2, 1-b) thiazolhydrochloride)	Broad spectrum anthelminthic	Methylene blue	1.0-3.0 ppm in water for 3-5 days.
		Metronidazole (1-beta (hydroxyethyl)-2-methyl-5-ni- troimidazole)	4 mg/liter of water for 3-4 days for con- trol of protozoan ectoparasites in orna- mental fishes.
Copper sulfate (Blue stone) CuSO₄ anhydrous CuSO₄ 5H₂O crystalline	For a 1 minute dip: 1:2,000 (500 ppm); in hard water add 1 ml glacial acetic acid /liter. 0.25-2 ppm to ponds. Quantity depends on hardness of water. Hard water requires more.	Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo- 1,8-naphthyridine-3-carboxylic acid) NegGram; Wintomylon.	Similar in action to oxolinic acid. One tablet per 50 to 100 liters of water for treatment of 3-4 days duration. Infec- tions with gram-negative bacteria.
Cutrine (chelated copper compound)	Aquatic herbicide as copper sulphate but not affected by hardness of water, and somewhat less toxic to fish.	Neguvon	See Dylox
		Ni-furpirinol	See Furanace (P-7138)
Cyzine (Enheptin-A)	20 ppm in feed for 3 days for Hexamita	Nifurprazine (HB-115) (Nitrofuran, unstable in prolonged exposure to sunlight. 1-(5 nitro-2-furyl) -2-(6-amino-3-pyridazyl) ethylene- hydrochloride) (Carofur and Aivet are water soluble formulations.)	As bath: for indefinite period 0.01-0.1 ppm. In food: 10 mg/kg of food. Feeding for 3-6 days at a time.
Detrapan (in use in France)	Systemic antifungal drug, for fish after spawning: 0.25 ml/kg intramuscularly used twice every 48 hours		
Devermin	0.1 g/kg of fish orally with food for con- trol of Cestoda.	Nitrofurazone (5-nitro-2-furaldehyde semicarbazone)	See Furazolidone, Furoxone
Diibutylin dilaurate (Butynorate, Tinostat)	250 mg/kg of fish orally or 0.3% in food	Oxolinic acid (1-ethyl-1, dihydro-6, 7-methyl- enedioxy-4-oxo-3-quinoline carboxylic acid)	For control of Aeromonas infections. Orally 3 mg/kg fish once daily for 5 days. As bath: 1 ppm for 24 hours for columnaris disease.
Dimeton	See Sulfamonomethoxine		
Dimetridazole	0.15% mixed with food for 3 days	Oxytetracycline (Terramycin)	50-75 mg/kg body weight per day for 10 days with food. (Law requires that it must be discontinued for 21 days before fish are killed for human consumption.)
Dipterex	See Dylox		
Diquat (patented herbicide, Ortho Co. con- tains 35.3% of active compound)	1-2 ppm of Diquat cation, or 8.4 ppm as purchased added to water. Treatment for 30-60 minutes. Activity much reduced in turbid water.	Ozone	Is being investigated as remedy for exter- nal infection and for decontamination of water.
Dylox (Dipterex, Neguvon, Chlorophos, Tri- chlorfon, Foschlor, Masoten)	0.25 ppm to water in aquaria and 0.25-1.0 ppm in ponds for indefinite period	Potassium permanganate KMnO₄	1:1,000 (1,000 ppm) for a 10-40 second dip. 10 ppm up to 30 minutes, 3-5 ppm added to aquarium or pond water for indefinite time.
Enheptin (2-Amino-5-nitrothiazole)	0.2% in food for 3 days for Hexamita	Potentiated sulfonamide (Sulfadimethoxine potentiated with ormetoprim)	For control of furunculosis and other sys- tematic infections. Used with feed 50 mg /kg fish per day.

66

Table 1 continued.

Chemical agent	Method of administration	Chemical agent	Method of administration
Povidone-Iodine (PVP-1)	See Iodophors	Sulfamerazine (cont.)	for 14 days. (Law requires that treatment must be stopped for 21 days before fishes are killed for human consumption.)
Quinine hydrochloride or Quinine sulfate	10-15 ppm in water for indefinite time	Sulfamonomethoxine (trade name Dimeton; water soluble)	With feed as is at a rate of 100-200 mg/kg of feed. Use as needed.
Roccal (Sold as 10-50% solution of Benzalkonium chloride. Quaternary ammonia germicide—also see Hyamine)	1-2 ppm in water for 1 hour. Toxic in soft water: less effective and less toxic in hard water.	Sulfisoxazole (Gantrisin)	200 mg/kg body weight per day with food
Sodium chloride (table salt, iodized or not)	1-3% in water for 30 minutes to 2 hours only for freshwater fishes.	Terramycin	See Oxytetracycline
Sulfadimethoxine sodium (in Japan available as 10% powder)	100-200 mg calculated as pure drug per kilogram of food	Tetrafinol	For control of intestinal helminths, used with feed.
Sulfamerazine	200 mg/kg body weight per day with food	Tin oxide, di-n-butyl	See Butyl tin oxide
		Wescodyne (Iodophor containing 1.6% of iodine)	Use as explained under Iodophors

Chemoprophylaxis

With fishes, drugs are used for prevention (chemoprophylaxis) and treatment (chemotherapy). Chemoprophylaxis is very effective, particularly if applied when an outbreak of a particular disease is anticipated. Outbreaks of diseases in fish farms are greatly affected by environmental stress, and chemoprophylaxis is very effective provided the stress factor is removed before the treatment ends.

In some chronic diseases, such as corynebacterial kidney disease, timely use of chemoprophylaxis with sulfonamides may prevent, or reduce, losses very significantly. In endemic areas, fish should receive sulfamethazine or sulfamerazine with feed at a rate of about 4 g/100 kg fish per day. This treatment may be repeated daily, given several days in a week, or repeated periodically. Usually, such treatment is continued for months (Herman, 1970).

The danger of chemotherapy is in developing strains of bacteria which are resistant or contain the transferable resistance factor "R". Microorganisms isolated from imported ornamental fishes often contain a wide spectrum of transferable resistance factors (Gratzek, 1978). There is indirect evidence showing that these fish were treated with various drugs.

Prophylaxis is now applied for removal of pathogens which may be present on the surface of fish eggs. Various chemicals were used for this purpose, but recently these have been replaced by iodophors, complexes of iodine and organic chemicals. Eyed eggs are usually treated with iodophors by immersion for 15 minutes in water buffered to about pH 7.0-8.0 and containing about 100 ppm of elemental iodine present in the iodophor. It has been shown that iodophors are not only effective in control of external bacteria, but also in viral contamination (Amend and Pietsch, 1972; Nelson, 1974a).

Prophylaxis is very important in protecting incubating fish eggs from the fungus *Saprolegnia*. One of the most reliable and most widely used methods of control is the exposure of eggs (at 1- to several-day intervals) to a bath containing 2-5 ppm of malachite green (Nelson, 1974b).

Parasite Control

There is a wide selection of therapeutic agents for the control of external and intestinal parasites of fishes (Hoffman and Meyer, 1974). However, there are no treatments for systemic parasites. The intradermal parasites such as *Ichthyophthirius* and *Cryptocaryon* are very bothersome. Only their free-swimming stage is amenable to drugs. It is difficult to reach the disease-causing stage of these parasites which are buried in the skin. Observations incidental to research on potentiated sulfonamides (Bullock et al., 1974; McCarthy et al., 1974) have shown that the potentiator ormetoprim accumulates in the skin of fishes. It would be interesting to find out if it has any effect on the intradermal form of these parasites.

Early Diagnosis Important in Treatment

Chemotherapy and other treatment methods have recently been reviewed by Herman (1970) and Hoffman and Meyer (1974). Therefore, I will make only general comments here. To be effective, chemotherapy must be prompt and directed toward the specific pathogen. Therefore, correct diagnosis is of utmost importance. When diagnosis must be delayed for a day or two, it is desirable to make a tentative diagnosis immediately and start treatment because any delay may increase losses considerably. The selection of the drug may have to be modified when the final diagnosis is made and the drug's susceptibility to the pathogen is determined.

Effectiveness of Chemotherapy

In the evaluation of the effectiveness of chemotherapy, counts of mortalities are important. One must remember that reduction of mortalities may not be real but only apparent by additive counting of losses. Whenever possible, mortalities should be expressed as mortalities per day, or per period, and based on the number of fish surviving at the start of each period. This calculation is only possible if a fairly accurate number of fish is known before the disease breaks out and if accurate daily counts of losses are made.

COST OF DISEASE CONTROL OF PRIME IMPORTANCE

Realistically speaking, the monetary value of losses caused by diseases is limited. Therefore, the cost of disease control cannot exceed the value of lost fishes. It is well to keep this in mind when developing methods for control of fish diseases.

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Fish Disease Inspection and Certification

DIANE ELLIOTT

ABSTRACT—Many countries now require that incoming shipments of salmonid fishes be inspected and certified free of certain diseases. This paper briefly describes general procedures for conducting disease inspections of salmonid rearing facilities. In addition, methods for detecting the presence of bacterial pathogens, the sporozoan Myxosoma cerebralis, *and three viruses (infectious pancreatic necrosis, infectious hematopoietic necrosis, and viral hemorrhagic septicemia) are outlined. Modifications of inspection procedures are also discussed.*

The growing awareness of the spread of fish diseases by indiscriminate transfers of fish has led to the development of laws and regulations controlling the importation of fish, fish eggs, and some fish products in at least 42 countries. Some countries, particularly the United States, Canada, and a number of others, require that incoming salmonid fish shipments or the hatcheries of origin be examined by a recognized hatchery inspector and certified free of certain diseases. In addition, some U.S. States and Canadian Provinces require that fish shipments imported from other states or provinces within the country also be inspected.

Most of the inspection and diagnostic procedures which I will describe pertain only to salmonid diseases. The methods for the most part are those which were approved by the Fish Health Section of the American Fisheries Society (1975), and are now available as a manual. Therefore, I will not go into each procedure in detail; instead, I will concentrate on some of the problems that I have encountered when using these procedures or some of the changes which I have made to fit certain needs.

DISEASE-FREE CERTIFICATION OF FISH

For a fish rearing facility to be certified free of a specific disease or diseases, periodic inspections must be conducted by a qualified biologist. Frequency of inspections and the number of inspections required before disease-free certification can be issued varies from nation to nation, but inspections are usually conducted once or twice yearly with two to four samplings required prior to issuance of disease-free certification. Issuance of this certification is also based on the hatchery owner's warranty that no uncertified fish or eggs have been brought to the hatchery subsequent to inspection and no major undiagnosed mortalities have occurred since inspection.

During an inspection, it is imperative that all lots of fish present at the facility be sampled by the certifying inspector. A lot is defined as fish of the same age which have always shared the same water supply and which have originated from a discreet spawning population.

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Sampling

After determining the number of lots in a hatchery, the next step is to decide on the number of fish to be sampled from each lot. In an inspection, the hatchery inspector is attempting to detect asymptomatic carriers as well as diseased fish. Therefore, the method of determining the actual number of fish to be sampled is based on obtaining a 95 percent probability of detecting at least one disease agent carrier in a lot with an assumed prevalence of carriers. According to American Fisheries Society procedures, the assumed carrier incidence is 5 percent for all diseases which are detectable in the carrier state except infectious hematopoetic necrosis (IHN) virus where the assumed incidence is 2 percent. Some other countries have slightly different procedures based on different assumed carrier incidences. Tables are available to help the inspector determine the correct sample size for each lot of fish. Once the correct sample size has been determined, the inspector must follow approved procedures for selecting tissues to be sampled and for processing the sample tissues. The procedure, used, of course, depends on the disease agent or agents which the inspector is trying to detect.

Viral Diseases

The pathogens which I have most commonly inspected for are the three salmonid virus disease agents: viral hemorrhagic septicemia (VHS), infectious pancreatic necrosis (IPN), and IHN. At present, the only approved procedure for determining the presence or absence of any of the viruses involves the isolation of the virus from fish tissues or sex products on cell culture. For detection of IPN and VHS viruses, I sample the posterior kidney and spleen from fish over 5 cm in length, entire viscera from fingerlings up to 5 cm, and for sac fry, the entire fish. For IHN virus, the only reliable way to detect the carrier state is to sample the ovarian or seminal fluid of spawning fish. Ovarian fluid is preferred for most fish species except for rainbow trout where seminal fluid has been found to carry the virus to an equal extent. Samples for virological analysis are transported to the laboratory on ice,

but are never frozen, because some IPN virus strains lose viability after freezing and thawing. Visceral samples are processed by first homogenizing in saline and then centrifuging the homogenate to remove debris and bacteria. Total dilution of visceral samples prior to inoculation should not exceed 1:200. The ovarian fluid samples are usually processed like the tissue samples, except that they are preferably not diluted, or at the most not more than 1:10.

Samples are then decontaminated by adding penicillin, streptomycin, and mycostatin. Gentamicin[1] may be substituted for the penicillin and streptomycin and it may be preferable since it inhibits some of the mycoplasmas and is more efficacious against some of the pseudomonads. Pseudomonads sometimes give a false cytopathic effect (CPE) reaction which resembles that of IHN. After a 2-hour period at 15°-20°C to allow the antibiotics and antimycotics to work, the samples are refrigerated until inoculation. The samples must be inoculated into cell cultures within 7 days after they are collected.

I use the rainbow trout gonad (RTG-2) cultures for IPN detection, and fat head minnow (FHM) cultures for IHN and VHS detection. Some inspectors use only one cell line for all viruses, which is acceptable, but I use two lines because I have found that the RTG-2 line is much more sensitive to IPN virus and the fat head minnow line seems to be a little more sensitive to the IHN virus. In addition, the use of two lines in many cases for one sample gives a double chance for virus isolation, particularly if something should go wrong with one set of cultures. Both of these cell lines are stable, have been karyotyped, and are commercially available. All of my cell cultures are grown in Eagle's MEM with Earle's base and with 10 percent agamma newborn calf serum added. Fetal calf serum may be used with good results. I use the new Falcon Multiwell Plate or Linbro Multi-Dish Tray rather than conventional tissue culture tubes. Each of the

24 wells in the plates easily hold about 1 ml of media, but the plates occupy much less incubator space and are quicker to inoculate and read than are tubes.

The plate may be handled as a closed system by using an adhesive film to cover the wells, or as an open system by using the lid which comes with the plate. To maintain the pH in the open system, I use Tris buffer. Samples are inoculated into cell cultures when the cultures are at least 24 hours old, but not more than 72 hours. When the cell monolayers are 80 to 90 percent confluent, 0.1 ml of each sample is inoculated into each of two cell cultures. Positive and negative controls are included on each culture plate or on each set of culture plates for one cell line. The inoculated cultures are incubated at 15°C for all three viruses and are observed for at least 14 days for the development of viral CPE. Material from any suspicious-looking cultures is inoculated into fresh cultures. If the CPE resembles that caused by any of the three salmonid viruses, a serum neutralization test is performed using specific antiserum to confirm the identity of the isolate. I usually use serum produced in either trout or rabbits for this purpose.

PARASITIC DISEASES

The only parasite which I have routinely inspected for is *Myxosoma cerebralis,* the causative agent of whirling disease. To detect this parasite, I use the method developed by Markiw and Wolf (1974). The inspector samples heads of fish between 4 and 12 months of age, as this is the age where the fish are still small enough to handle easily, but are old enough to be carrying spores rather than just trophozoites. The fish are processed in pools of up to 60 fish. The heads are heated for 5 to 10 minutes in water at 50°C to make flesh removal easier. The flesh, brain, spinal column are discarded, retaining the skull and gill arches. These elements are digested in acidic pepsin solution at 37°C until no chunks remain. The mixture is centrifuged at 1,200 g for about 10 minutes. The pellet is then further digested in basic trypsin solution at

22°C for 30 minutes. After stopping the trypsin digestion by adding serum, the material is centrifuged again and the pellet is resuspended in a small amount of saline with serum. It is layered on top of a 55 percent dextrose solution and centrifuged at 1,200 g for 30 minutes. If the spores are present, they will be found in a pellet at the bottom of the dextrose gradient. The efficiency of spore recovery with this method, according to Wolf, is about 80 percent.

I have found this method to be quite satisfactory for the most part, but have experienced one problem with it. I have seen some false positives in certain areas in the State of Washington where coho and chinook salmon carry a sporozoan, *Myxobolus kisutchi,* in the hind brain and spinal cord (Yasutake and Wood, 1957). The parasite looks very much like *Myxosoma cerebralis.* Although we remove as much of the brain and spinal column as we can when processing the fish, if the incidence of this parasite is very high, spores frequently will remain. There are criteria to distinguish between the two parasites. For example, *Myxobolus* has an iodinophilous vacuole, while *Myxosoma* does not. This can be determined by staining viable spores with an iodine preparation and examining microscopically. Another procedure which can be used is to examine the head and spinal cord area histologically to determine the location of the parasite. *Myxobolus kisutchi* occurs in the brain and spinal cord only, and not in the cartilaginous or bony elements of the head and spinal column where *Myxosoma cerebralis* occurs.

BACTERIAL DISEASES

I have been asked occasionally to inspect for the presence of certain bacterial diseases. If the hatchery is being inspected for certification, I usually select only moribund fish from the hatchery over a period of several months. I sample by streaking material from the kidney or other organs which exhibit lesions onto the appropriate agar medium. I also gram stain the material and look for bacterial pathogens. Isolated organisms are identified primarily by biochemical testing, since serological reagents have only recently

become available. Standard media are used in conjunction with the API Analytab system. This system was designed for the identification of Enterobacteriaceae and other gram-negative organisms, such as *Pseudomonas, Aeromonas, Vibrio,* and *Flavobacterium*. Since most fish pathogens are gram negative, the system works fairly well for identifying them. The API Analytab contains dehydrated media for over 20 different biochemical determinations. The media is reconstituted by adding distilled water or saline in which the colony to be identified is suspended. The colony is picked from a culture plate for this determination. This system is well suited to a small laboratory which may not be able to produce many kinds of biochemical test media. One of the main advantages of the API Analytab, besides its compact size and ease of use, is the coding system which has been developed by API for quick and easy identification of the organisms. The system is designed primarily for human pathogens, rather than fish pathogens, but a number of fish organisms, such as *P. flourescens* and *A. liquifaciens,* are included. In addition, I have modified the coding to identify other fish pathogens.

I should mention that for the bacterial kidney disease organism (*Corynebacterium* sp.), which is not very amenable to culturing, I have been relying up to this point strictly on gram stain characteristics. Recently, immunodiffusion and indirect flourescent antibody techniques have been developed to detect the presence of the kidney disease organisms in fish. Both of these methods are much more sensitive than the old gram stain method, and both are described in the American Fisheries Society (1975) disease detection procedures manual. The flourescent antibody technique is a little more suited to our needs since it takes about 1-2 hours to complete, as opposed to 20-24 hours for the immunodiffusion technique.

DIAGNOSTIC EXAMINATION OF FISH

For diagnostic purposes, as opposed to disease-free certification, 25-50 diseased fish are requested. I prefer to receive the fish while they are still alive. If this is not possible, I request that they be sent on ice, but not frozen, or that some be fixed in Bouin's solution and the rest sent on ice. When the live fish are received, they are divided into four groups. Some are examined externally and internally for parasites, some are prepared by bacteriological analysis, some are processed for virological analysis, and the rest are placed in Bouin's fixative for histological sectioning. The use of four methods of analysis provides a good picture of the disease pathology and its etiology.

MOVING TOWARD IMMUNOLOGICAL TECHNIQUES

The diagnostic and inspection field is moving more and more toward immunological techniques such as serum neutralization, immunodiffusion, immunoflourescence, and immunoperoxidase for the detection and identification of fish disease agents. These methods should help to revolutionize the field, as they are much more sensitive and rapid than many of the older techniques.

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Foreign Catch Cut in
1978 Fishing Regulations

Regulations published by the Commerce Department's National Oceanic and Atmospheric Adminstration will reduce the amount of fish allocated to foreign fishermen from the 1977 level of 2.1 million metric tons (t) to 1.9 million t.

Changes to regulations controlling foreign fishing within the U.S. 200-mile conservation went into effect 1 January according to NOAA's National Marine Fisheries Service.

The reduction is caused by changes in the amounts of some species that can be caught and still permit the stocks to grow, and an increase in the capability of American fishermen to catch more fish.

Another major change in the 1978 regulations provides for an in-season review of fishing to determine if adjustment should be made in the total allowable catch for each species, in the estimates of domestic catch, and in the amount of fish that may be caught by foreign fishermen. The review will be based primarily on current information on the status of the stocks, and actual versus estimated performance of domestic and foreign fleets in current and prior years.

The largest reduction in the amount that may be allocated to foreign fishermen occurs off the northeast coast. Amounts of six species available have been established as follows (1977 allocations are in parenthesis): silver hake - 45,400 t (85,500); red hake 27,400 t (34,900); Atlantic herring - 0 t (22,000); Atlantic mackerel - 1,200 t (69,000); butterfish - 4,000 t (5,500); and other finfish - 46,800 t (60,000). Squid - 42,500 t and river herring - 500 t remain unchanged from 1977.

Other changes affecting the northeast area are the establishment of a single foreign fishing area or "window" rather than several windows which were established in 1977. A quota has been set on the amount of each species that a foreign country is permitted to catch. When the quota is reached for any one of the species, all fishing by that country will be prohibited even though the quotas on other species may not have been reached.

All nets used by foreign fishermen on the bottom must have meshes no smaller than 60 mm, and all other nets must have meshes no smaller than 45 mm.

Off the Pacific coast, the amounts of five species available to foreign fishermen have been reduced: Pacific hake - 89,000 t (123,200); rockfishes, including Pacific ocean perch - 710 t (1,600); flounders - 90 t (125); sablefish - 90 t (250); other species - 445 t (600). The latter four species may only be taken as a small, unavoidable by-catch percentage of a nation's allocation of Pacific hake. The amount of jack mackerel available remains at 4,000 t. Foreign fishermen in the area were restricted to a "vessel-day" limitation as well as an allotment last year; however, this limitation has been removed because its value as a management technique in this fishery is uncertain.

In the Gulf of Alaska, available amounts of five species of fish have

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Shellfish Supplies Temper Prices

Supplies of some major shellfish products—shrimp, scallops, lobster tails, and west coast crab—increased in the first half of 1977 over average supplies of the previous 2 years, according to the National Oceanic and Atmospheric Administration's National Marine Fisheries Service, a Commerce Department agency. Greater supplies brought some softening in prices, viewed as a normal market reaction and not as an indication of any underlying weakness in demand.

Sharp price increases experienced in 1975 and 1976 likely have affected the demand for shellfish, fisheries officials believe, making prices more sensitive to changes in supplies. Thus, even though strong market demand for shellfish products is anticipated through the first half of 1978, supplies of some major shellfishes are expected to be sufficiently high to influence prices. The prices of shrimp, lobster tails, and king crab could drop, while higher prices are likely for scallops and American lobster, supplies of which are not likely to increase, officials believe.

Record landings of snow crab in 1977 resulted from increased fishing effort, while the rise of landings of shrimp and scallops was associated with improved resources. In the first half of the year, imports of shrimp and scallops were at record levels. Imports of lobster tails have increased from countries which, in the past have supplied relatively small amounts to the United States. Receipts from Nicaragua, for example, doubled from the previous year. Copies of "Shellfish—Market Review and Outlook—November 1977" are available from the Industry and Consumer Services Division, National Marine Fisheries Service, NOAA, Washington, DC 20235.

been reduced, and four have been increased: pollock - 117,340 t (149,000); Pacific Ocean perch - 18,900 t (29,000); flounders - 17,600 t (20,500); sablefish - 8,000 t (19,500); other species - 12,960 t (16,200).

Those increasing are rockfish - 4,080 t (4,000); Atka mackerel - 24,800 t (22,000); Pacific cod - 16,980 (2,300); squid - 1,600 t (0). An additional amount of fish, equivalent to 20 percent of the total allowable catch for each species, has been held in reserve for later allocation to foreign nations if U.S. fishermen do not exceed their estimated catch.

The amounts available in the Aleutian Islands and Bering Sea have changed also. Of the species available to foreign fishermen, six remain unchanged, two have increased, and three have decreased. Unchanged are pollock - 950,000 t, yellowfin sole - 106,000 t, Pacific Ocean perch - 21,500 t, squid - 10,000 t, other species - 93,600 t, and snails - 3,000 t.

Those species which have larger amounts available are: other flounders - 139,000 t (105,000); Atka mackerel - 24,800 t (0). The initial amounts available have been reduced for: sablefish - 3,900 t (7,400); cod - 58,000 t (56,500); and herring - 8,670 t (20,000). The amount of tanner crab available was to be decided at a later date.

A reserve of 1,500 t of Pacific cod and 600 t of sablefish in the Bering Sea has been established for possible allocation at a later date.

In the Western Pacific 2,000 t of seamount groundfish are available.

The regulations are published under the authority of the Fishery Conservation and Management Act of 1976 and continue in effect until either amended by the Service or superseded by regulations implementing fishery management plans developed by the eight U.S. Regional Fishery Management Councils.

NOAA Names Three Top-Level Appointments

Three appointments to top-level positions at the National Oceanic and Atmospheric Administration have been announced by Richard A. Frank, NOAA Administrator.

William C. Brewer, Jr., who has served since 1974 as NOAA General Counsel, will become Special Representative of the Secretary of Commerce to the Law of the Sea Conference and Special Representative of the NOAA Administrator on Law of the Sea and International Law. Eldon Van Cleef Greenberg, Deputy General Counsel of the Agency for International Development, will become General Counsel of NOAA. Samuel A. Bleicher, who has been Acting Assistant Administrator for Policy and Planning, will become Director of the newly-created NOAA Office of Ocean Management of the Commerce Department agency.

Brewer, a resident of Washington, D.C., was a partner in the Boston law firm of Hill and Barlow before his appointment as General Counsel of NOAA in 1974. He was graduated from Phillips Academy in 1939, Williams College (Phi Beta Kappa) in 1943, and from Harvard Law School in 1949. At that time he became General Counsel of the Mutual Boiler and Machinery Insurance Company. In 1953 he became a partner in the law firm of Peabody, Koufman and Brewer, which was succeeded by the firm of Hill and Barlow in 1965.

Brewer has served as adjunct professor at Boston College Law School, teaching coastal zone management and international business law. He has been a director of several companies, and has written numerous articles for legal journals.

Greenberg, who also lives in Washington, D.C., was graduated Magna Cum Laude from Harvard College in 1965 with a Bachelor of Arts degree. After post-graduate studies at the Institut de'Etudes Politiques de Paris in France from 1965 to 1966, he took his J.D. degree Cum Laude from Harvard Law School in 1969.

From 1969 to 1970 he was law clerk to Edward C. McLean, U.S. District Judge for the Southern District of New York, and for the next 2 years was associate attorney at Debevoise, Plimpton, Lyons and Gates of New York. He was staff attorney at the Center for Law and Social Policy in Washington, D.C. from 1972 to 1977 when he became Deputy General Counsel at the Agency for International Development.

The author of several articles appearing in legal journals, Greenberg has served as adjunct professor in administrative law at Georgetown University Law Center. He was Advisor to the United States Delegation to the Law of the Sea Conference from 1975 to 1977.

Bleicher, a resident of Toledo, Ohio, was graduated from Northwestern University in 1963, Phi Beta Kappa, with Honors in Economics, and received his J.D degree from Harvard Law School in 1966. He is a law professor at the University of Toledo College of Law, with emphasis on environmental, constitutional, and international law. He served from 1972 to 1975 with the Ohio Environmental Protection Agency, first as Deputy Director for Regulation and then as Deputy Director for Regulation and Enforcement. In 1976 and 1977 he served as Issues Analyst for the Carter-Mondale Presidential Campaign and Carter-Mondale Transition Team. He joined NOAA as Special Assistant to the Administrator with responsibilities for reorganization, policy planning, and executive recruitment in February 1977. He is the author of articles in several law journals as well as college course materials on pollution and political boundaries.

New Chart, Bathymetric Map Catalog Published

A new catalog of special purpose charts and bathymetric maps has been published by the National Oceanic and Atmospheric Administration (NOAA).

Produced by the Commerce Department agency's National Ocean Survey, the five-color map and chart catalog indexes bathymetric maps, topographic/bathymetric maps, and marine boundary maps and charts.

Also indexed for the first time are Tidal Current Charts, Marine Weather Service Charts, Storm Evacuation Maps, and orthophoto maps of Florida's coastal zone. Included are insets indexing Offshore Mineral Leasing Area Maps, Geophysical Maps, and the International Hydrographic Bureau (IHB)-supported General Bathymetric (plotting) Charts of the Oceans (GEBCO).

The catalog displays the new Fishery Conservation Zone with the 200-m (Continental Shelf) and the 2,500-m (Continental Slope) zones. A list of map and chart definitions describes each of the various products available, map and chart purchasing instructions, and prices.

The catalog, "Map and Chart Catalog 5 - Bathymetric Maps and Special Purpose Maps," is available free to the public. It may be obtained from NOAA's National Ocean Survey, which made copies available at several boat shows early in the year. The catalog also may be obtained from the National Ocean Survey, Distribution Division (C44), Riverdale, MD 20840 (Phone: (301) 436-6990); Counter Sales, National Ocean Survey, 6001 Executive Boulevard, Room 101, Rockville, MD ((301) 443-8005); or from local marine supply agents.

NOAA, FWS Will Aid Water Users

The Commerce Department's National Oceanic and Atmospheric Administration (NOAA) and the Interior Department's Fish and Wildlife Service (FWS) have signed an agreement expected to provide improved services to the country's landowners and land and water users. The agreement, by NOAA Administrator Richard A. Frank and FWS Director Lynn A. Greenwalt, establishes formal cooperation between NOAA's Marine Advisory Service programs and those of the Fish and Wildlife Service, especially in such activities as wetlands research and assistance to water users.

Cooperation, according to the agreement, will include such areas of mutual concern as the preparation of educational and research programs, the dissemination of information to the public, and the exchange of data routinely collected by field agents in the course of their research.

A similar agreement was signed late last year between the Fish and Wildlife Service and the Agriculture Department's Extension Service.

Cooperation among the three federal agencies is not new. The Fish and Wildlife Service has been active in working with the Extension Service for more than 40 years, and there has been considerable cooperation between the Marine Advisory Service and Extension Service ever since the former was created in 1966.

The creation of the formal agreement, however, is taken as a salutory sign by those concerned with America's water resources. For the first time there will be an institutional arrangement bringing together experts in the Fish and Wildlife Service with their counterparts in Land and Sea Grant universities in the 50 states, Puerto Rico, Guam, the Virgin Islands, and the District of Columbia.

Foreign Fish Vessels Off U.S. Coastlines Decrease in October

The number of foreign fishing and fishing support vessels sighted off U.S. coasts in October was 378, a decrease of 59 from the 437 sighted in September, according to preliminary figures released by the National Oceanic and Atmospheric Administration's National Marine Fisheries Service, a Commerce Department agency.

The 378 vessels sighted compare with the 452 sighted off our coasts in October of 1976. The decrease is due primarily to the closure of the hake, squid, and mackerel fisheries off the Atlantic coast. Normal seasonal decline in fishing activities and reduction in the number of foreign vessels permitted to fish within the 200-mile zone also contributed to the reduction.

The foreign vessels, from six nations, were sighted off the coasts of New England and the mid-Atlantic States, West Coast, and Alaska. The largest number, 273, was from Japan, which had 272 vessels fishing for groundfish and pollock off Alaska, and one tuna longliner fishing in the western Pacific. The Soviet Union had 75 vessels: 45 fishing for pollock in Alaskan waters, and 30 fishing for hake off the Pacific coast.

A summary of foreign vessels operating off U.S. coasts during October 1977 and October 1976 follows:

		No. of vessels	
Area	Nation	Oct. 1977	Oct. 1976
New England, mid-Atlantic	E. Germany	0	25
	Soviet Union	0	30
	Poland	3	9
	W. Germany	1	7
	Spain	0	23
	Japan	0	26
	Italy	0	5
		4	125
Gulf of Mexico	Panama	0	5
West Coast	Panama	0	3
	Japan	1	0
	Soviet Union	30	9
	S. Korea	0	10
	Bulgaria	0	6
	Poland	3	6
	E. Germany	0	6
	Canada	1	[1]0
	Taiwan	0	1
	Liberia	0	1
		35	42
Alaska	Japan	272	187
	S. Korea	21	48
	Taiwan	0	3
	Soviet Union	45	42
	Poland	1	0
		339	280
	Total	378	452

[1]Number of Canadian vessels off U.S. shores not recorded.

Foreign vessels sighted off the coasts in 1976 were as follows: January-420, February-510, March-435, April-560, May-924, June-970, July-842, August-543, September-514, October-452, November-258, December-240. In 1977: January-319, February-314, March-180, April-235, May-374, June-767, July-786, August-492, September-437, and October-378.

Japan Makes Major Change in Fisheries Foreign Aid Program

Japanese foreign aid program in fisheries endeavors is implemented through several semi-governmental entities. The Japan Overseas Fisheries Cooperation Foundation is one of the largest such nonprofit making public corporations. The Foundation was established in June 1973 as a result of a strong campaign by the Japan Fisheries Association. The Association itself has a semi-governmental character, although, it is an industry lobby group, representing nearly all segments of the Japanese fishing industry, including giant corporations and federations of small cooperative associations consisting of artisanal fishermen.

In July 1972, 5 years before the establishment of the U.S. 200-nautical-mile fisheries conservation zone, the

Association drafted a bill to develop a measure to counter the effects to be created by such extended jurisdictions. The bill was never enacted, but several of the measures it proposed were forthwith implemented.

Thus, in June 1973, the Japan Overseas Fisheries Cooperation Foundation was established. The Foundation has been mandated to aid the fisheries of foreign nations in which Japanese fishermen fish, so that both the coastal nations and the Japanese industry would benefit.

The purposes of the Foundation are fivefold: 1) To loan low interest, long-term funds to Japanese-owned or controlled fishing companies located overseas. 2) To train Japanese fisheries experts for overseas aid assignments. 3) To invite foreign fisheries trainees and leaders to visit Japan, or to be trained in Japan. 4) To aid in bilateral fisheries negotiations. 5) To station Japanese fisheries experts in coastal nations for technical development aid.

The Foundation has performed all of these functions with increasing vigor since its inception. Especially noteworthy has been the increase in the amount of money placed in low-cost loans. Initially, the Foundation's endowment amounted to 200 million yen, (US$667,000)[1], obtained in equal amounts from the Government and the private industry. At the same time, one billion yen (US$3.33 million) of the Foreign Ministry budget for FY73 (1 April 1973 through 31 March 1974) was set aside for use by the new aid corporation to loan during that fiscal year.

During its first 4 years of operation (through 31 March 1977) the Foundation placed 8.49 billion yen (US$35.4 million) in loans to contractors engaged in a total of 34 projects around the world. In FY 1977 the Foundation plans to place 5 billion yen (US$20.1 million)[2] in loans, and in FY 78 alone, it plans to grant loans totaling 8 billion yen (US$33.3 million).

Along with this sharp rise in the amount of loans placed, a significant change is taking place in the Foundation's loan management. Until recently, only Japanese nationals were entitled to the low-cost loans from the Foundation. But, in October 1977, the Foundation decided to place a loan directly to the Government of Argentina.

A consortium of five Japanese fishing companies had successfully bid for the development of the fisheries in the Patagonian region of Argentina. A part of the agreement for the development was that the consortium would have two research vessels constructed according to the Argentinian specifications, and would turn them over to Argentina. However, the Foundation intervened to grant a direct loan to the host government for the construction of the vessels. In the board meeting of the Foundation held on 29 November 1977, the trustees allowed the Foundation to loan directly to host countries, thus officially introducing a significant change in the Foundation's policies. The Japanese fishing industry leaders are reportedly very pleased with the new direction the Foundation's aid program has taken. (Source: LSD 78-1.)

Corral-Grown Bluefin Tuna Sold in Japan

Bluefin tuna raised in a corral in southern Japan from baby fish weighing less than 1 kg each since 1974, made their first appearance for sale at the Tokyo Central Wholesale Market on 19 December 1977. The fish received a favorable rating by the buyers for their oil-rich meat and flavor. The corral-grown bluefin tuna ranged in size from 38 kg to 77 kg apiece, and were sold at prices between ¥3,900/kg and ¥5,000/kg (US$7.32/lb-$9.39/lb, at ¥242=US$1), averaging ¥4,600/kg ($8.64/lb). (Source: FFIR No. 78-1.)

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[1]According to the 1972 exchange rate.

[2]According to the December 1977 exchange rate.

Japan Eases Import Rules on Four Fishery Products

The Japanese Ministry of Agriculture and Forestry on 14 December 1977 announced plans to add smoked herring and cuttlefish to the list of liberalized import items and to lower import tariffs on shrimp and herring roe. The liberalization on smoked herring and cuttlefish was expected to take effect early in 1978. Import tariffs on shrimp dropped from the current 5 percent to 4 percent, and those on herring roe from 15 percent to 12 percent, on 1 April 1978.

All four items named in the announcement represent products for which Japan must depend heavily on foreign supply. Japan's domestic production of smoked herring shrank to 322 tons in 1975 and further to 133 tons in 1976 as a result of the total ban of Japanese herring fishery in the Soviet 200-mile zones in the Okhotsk Sea. Japan is currently importing about 60 percent of its demand for cuttlefish from Spain, the Canary Islands, and South Yemen. Imports of shrimp, the largest of all fishery items imported into Japan in value, amounted to 126,000 tons worth ¥223,100 million (US$921.9 million, at ¥242 = US$1) for 1976, and those of herring roe to 12,000 tons worth ¥28,900 million (US$119.4 million) for the same year. This is the first time in about 4 years that Japan expanded import liberalization to fishery products. (Source: FFIR No. 78-1).

ROK Claims Canned Oyster Export Lead

The Republic of Korea surpassed Japan to become the largest exporting country of canned oyster at the end of October 1977, announced a source close to canned oyster exporters in Seoul. South Korea's exports of canned oyster during the first 10 months of 1977 were worth more than US$1 million, exceeding by more than 50 percent 1977 export target of $11 million.

South Korea's successful expansion of canned oyster exports are attributed to the acceptance of the export prices and the consequent rise in demand for the Republic of Korea products in U.S., Australian, and European markets, according to the same source. By country of destination, the sale of South Korean canned oyster to the end of October 1977 was: $15 million (target $7 million) to the United States, $1.424 million to Australia, $0.344 million to Europe, and $0.833 million to other countries. (Source: FFIR No. 78-1.)

Japan's 1977 Saury Catch More Than Twice 1976 Mark

Saury landings by the Japanese coastal fishery up to 20 November 1977, reached 230,000 metric tons, more than double the comparable 1976 landings, according to the National Association of Saury Fishery. Because of the unusually heavy landings of small to medium-size fish measuring about 26 cm in length, the seasonal average price to 10 November remained low at ¥161/kg ($593/short ton at ¥246 = US$1), down 16 percent from the comparable 1976 average price. (Source: FFIR 77-16.)

USSR, Japan Conclude Bilateral Fishery Pact

On 16 December 1977, the Soviet Union and Japan signed in Moscow a protocol extending the USSR-Japan Fisheries Interim Agreement, and established 1978 catch allocations for each other, the NMFS Language Services Branch reports. The agreement allows the Soviet fishermen to catch 650,000 t of fish in Japanese 200-mile zone, and the Japanese fishermen to catch 850,000 t in the Soviet zone. The main species in the new Soviet allocations are sardine and mackerel, amounting to 318,000 t or nearly half of their total allocations, and the major species in the new Japanese allocations is Alaska pollock, taking up 40 percent or 345,000 t of the total Japanese allocations.

Japanese fishermen reportedly caught about 550,000 t of Alaska pollock in the Soviet zone from January 1977 to the present. The 1978 allocation for this species, being 40 percent below the actual catch for 1977, will necessitate the revocation of 17 medium trawler permits. Moreover, the Japanese crab and shrimp fisheries in the Soviet zone experienced similar reductions of allocations. Some permit revocations will be inevitable in these fisheries, also.

Japanese reports emphasize the significance of the 200,000 t difference between the two allocations. However, Japan has allowed the Soviets to maintain their historic catch level in the Japanese zone, but received in return only about 50 percent of its own historic catch in the Soviet zone. (Source: LSD 78-1.)

Fish, Harbor Work Get $18.4 Million in Canada

Canada's Fisheries and Environmental Department is spending $18,386,000 under the Federal Labor Intensive Projects (FLIP) program over the first nine months of 1978. This allocation, third largest among the 15 departments participating in the program, will be spent largely on fisheries management and small craft harbors projects, with lesser amounts on ocean and aquatic sciences and environmental management activities.

Fisheries and Environment Minister Roméo LeBlanc said the program will provide over 14,000 work-months of employment, particularly in the Atlantic provinces, Quebec, and in western Canada. Projects will range from the building and repair of fishing and recreational docking facilities to clerical and general labor activities at regional management and research centers.

LeBlanc said departmental FLIP allocations include $17,317,000 for the Fisheries and Marine Service—comprising $9,978,000 for small craft harbors, $6,177,000 for fisheries management, and $1,162,000 for ocean and aquatic sciences; also $939,000 for Environmental Management Service and $130,000 for Atmospheric Environment Service projects.